Predicting anti-arthritic drug effects in collagen-induced arthritis using short-term mechanistic models of collagen II immunity

Thesis

Predicting anti-arthritic drug effects in collagen-induced arthritis using short-term mechanistic models of collagen II immunity

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Abstract

Novel anti-arthritic drugs are often assessed in murine collagen-induced arthritis (CIA), which is a widely used pre-clinical model of rheumatoid arthritis. However, CIA studies are lengthy, development of arthritis is not synchronised and not all animals develop disease. Work conducted in this thesis addressed some of these issues by developing short-term mechanistic models of collagen II (CII) immunity. Drug effects on CII-induced hypersensitivity, anti-CII antibodies and *ex vivo* CII stimulated $CD4^+$ T cell proliferation in mice 14 days post-CII sensitisation were assessed and compared to their anti-arthritic effect in CIA.

As this thesis progressed, it was reported that IL-17 secreting $CD4^+$ T helper (Th17) cells represent a population of cells distinct from interferon gamma (IFNγ) secreting Th1 and IL-4 secreting Th2 cells and may be involved in autoimmune disease. However, the role of IL-17 and Th17 cells in CIA and CII immunity was not defined. The role of IL-17 and its relationship with Th1 and Th2 cells was investigated in the CII stimulated $CD4^+$ T cell assay. Results showed that CII specific Th1 and Th17, but not Th2 cells, are present in cultures of cells from CII sensitised mice. Addition of anti-IL-17 to these cultures increased the number of CII specific IFNγ secreting $CD4^+$ T cells. Literature evidence suggests that IFNγ is protective in CIA. This increase in IFNγ may therefore represent a novel mechanism of action by which anti-IL-17 exerts some of its anti-arthritic activity.

This thesis has shown that short-term models of CII immunity can predict anti-arthritic drug effects in CIA. A novel screening cascade has been proposed which could be used in drug discovery and may reduce the number of animals required for

CIA studies. Moreover, the differential effect of anti-arthritic drugs in these models suggests they can discriminate between drugs and identify novel mechanisms of action.

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Abbreviations

%	percent
μ	micro
α	alpha
β	beta
γ	gamma
°C	degrees Celsius
μm	micrometer
^3H	tritiated
Ab	antibody
ACR	American college of rheumatology
ANOVA	analysis of variance
APC	antigen presenting cell
APPs	acute phase proteins
AUC	area under the curve
BCIP	Indolylphosphate p-Toluidine Salt
BSA	bovine serum albumin
CD	cluster of differentiation
CFA	complete Freund's adjuvant
Ci	Curie
CIA	collagen-induced arthritis
CII	collagen type II
CO_2	carbon dioxide
cpm	counts per minute

CRP	C-reactive protein
DC	dendritic cell
DMARD	disease modifying anti rheumatic drug
DTH	delayed type hypersensitivity
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
ELISpot	enzyme-linked immunospot
ESR	erythrocyte sedimentation rate
Fab	fragment antigen binding
FACS	fluorescence activated cell sorter
Fc	fragment crystallisable
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
$Foxp3^+$	forkhead box protein 3^+
g	gram
GM-CSF	granulocyte macrophage-colony stimulating factor
gp	glycoprotein
h	hour
HLA	human leukocyte antigen
HRP	horseradish peroxidise
ICAM-1	intracellular adhesion molecule 1
IFA	incomplete Freund's adjuvant
IFNγ	interferon-gamma
Ig	immunoglobulin

IL	interleukin
L	litre
LFA-1	lymphocyte function associated antigen-1
M	molar
m	milli
mAb	monoclonal antibody
MACS	magnetic cell sorting
MHC	major histocompatibility complex
mm	millimetre
MMP	matrix metalloproteinase
MTX	methotrexate
n	nano
NBT	Nitro Blue Tetrazolium Chloride
NF-κB	nuclear factor kappa B
nm	nanometre
NSAID	non-steroidal anti-inflammatory drug
OD	optical density
OPG	osteoprotegerin
p	pico
PBS	phosphate buffered saline
PE	phycoerythrin
R	receptor
r	recombinant
RA	rheumatoid arthritis

RANK	receptor activator of nuclear factor kappa B
RANKL	receptor activator of nuclear factor kappa B ligand
RBC	red blood cell
RF	rheumatoid factor
RPMI	Rosweli Park Memorial Institute
s	soluble
s.e.m.	standard error of the mean
SAA	serum amyloid A
SCID	severe combined immunodeficiency
TCR	T cell receptor
TGFβ	transforming growth factor beta
Th	T helper T cell
TMB	3,3',5,5' tetramethylbenzidine
TNFα	tumor necrosis factor alpha
v	volume
VCAM-1	vascular cell adhesion molecule 1
VLA-4	very late antigen 4
w	weight
xg	acceleration relative to that due to the Earth's gravitational field

Contents

List of Figures

List of Tables

Chapter one

Introduction

1.1 Inflammation

The inflammatory response has evolved as a protective response to tissue injury. Tissue injury may occur as a result of trauma or chemicals, which generally gives rise to non-immune (innate) inflammation, or as a result of infection, which gives rise to a combination of non-immune and immune (adaptive) inflammation. Injury of the tissue results in the release of various chemical mediators from cells, for example histamine, eicosanoids, nitric oxide, neuropeptides and cytokines. The role of these mediators can change depending on the cellular environment and the pressure of other mediators to enhance or damp down the response as appropriate. Inflammation is therefore extremely complex.

Many inflammatory mediators have vasoactive properties and act together to dilate local blood vessels and to contract vascular endothelial cells to increase vascular permeability. The consequence of this is increased blood flow and leakage of plasma into the injured tissue. This process gives rise to heat, redness and swelling. The accumulation of plasma and cells at the site of inflammation also gives rise to pain through stimulation of sensory nerves. Heat, redness, swelling and pain are therefore the characteristic hallmarks of inflammation. Inflammatory mediators also stimulate the expression of cell adhesion molecules such as the selectins and intracellular adhesion molecule-1 (ICAM-1) on blood vessel endothelial cells. These adhesion molecules bind carbohydrate ligands and integrins respectively on circulating leukocytes allowing their extravasation into the injured tissue. The first cells to be recruited in an acute inflammatory response are polymorphonuclear granulocytes (neutrophils). These are followed by monocytes, which then differentiate locally into macrophages. Once in the tissue, these immune cells migrate along a chemotactic gradient to reach the site of inflammation, where they can attempt to remove the

injurious stimulus and repair the tissue. Persistence of the injurious stimulus gives rise to a state of chronic inflammation. Chronically inflamed tissue is characterised by the infiltration of mononuclear immune cells such as monocytes, macrophages, lymphocytes and plasma cells and is almost always accompanied by tissue destruction. The initial tissue damage or the reaction to it, which may be either temporary or permanent, can result in loss of tissue function. For a more detailed review of the inflammatory process refer to Rankin (2004).

1.2 Immune responses

The immune system is often divided into innate and adaptive immunity. However, the two systems overlap and should not be viewed in isolation. The innate immune response is initiated immediately after tissue injury. If the injurious stimulus is an invading organism (pathogen) then the innate and adaptive immune responses work together to eliminate the infection. The innate immune response occurs to the same extent each time the pathogen is encountered, whereas the adaptive immune response improves on repeated exposure to the same pathogen.

1.2.1 Innate immunity

Pathogen recognition by the innate immune system activates phagocytic cells (neutrophils and macrophages), other inflammatory cells (mast cells, basophils, eosinophils and natural killer cells) and activates the alternative pathway of complement. The innate response is not specific to the pathogen and is initiated by the recognition of conserved molecular patterns common to entire classes of pathogen by Toll like receptors (TLRs) on professional antigen presenting cells (APCs) such as dendritic cells or macrophages. Activation of TLRs results in the production of

chemokines and pro-inflammatory cytokines such as tumour necrosis factor alpha (TNFα) and interleukin (IL)-1. All these innate immune responses enhance the inflammatory process. In particular, the release of TNFα and interleukin IL-1 from activated macrophages increases the expression of cell adhesion molecules. These bind integrins on the surface of leukocytes aiding adhesion. Chemokines such as IL-8 are released from cells, such as macrophages, and direct large numbers of neutrophils to the site of infection. Activation of the complement system results in the production of a series of plasma proteins that coat pathogens. This process is known as "opsonisation" and marks the pathogen as a target for neutrophils and macrophages which then phagocytose and usually kill them. In addition, proteins from the complement system can act as chemoattractants and recruit cells to the site of inflammation and can form immune complexes that have direct lytic activity.

Cytokines released as a consequence of infection also act systemically and stimulate what is known as the "acute-phase response", which is another component of innate immunity. In this response acute-phase proteins (APPs) are produced and have a variety of actions in host defence. Many of these APPs are synthesised in the liver by hepatocytes (Selinger *et al.*, 1980) and are induced by the pro-inflammatory cytokines IL-1β, TNFα and IL-6 (Ramadori *et al.*, 1988). These proteins recognise conserved microbial structures and bind the invading organism. Some of these APPs such as C-reactive protein (CRP) act as opsonins and activate the complement cascade, which further augments opsonisation and pathogen elimination. In addition, some APPs act as chelators which slow microbial growth, while others are protease inhibitors which limit tissue destruction.

Innate immune responses either successfully clear infection or contain it while an adaptive immune response develops.

1.2.2 Adaptive immunity

The adaptive immune response conveys specificity towards the invading pathogen through recognition of non-self molecules or antigens. This response requires proliferation and activation of antigen specific B and T lymphocytes. Naïve B and T cells circulate through the lymphatic system where they encounter antigen and recognise it through cell surface receptors.

T cell receptors (TCR) recognise antigen presented on the surface of cells in association with major histocompatibility complexes (MHC). There are two classes of MHC molecule, class I and class II. MHC class I molecules are expressed on all nucleated cells, whereas MHC class II molecules are expressed on antigen-presenting cells (APCs) such as dendritic cells (DCs), B cells and activated macrophages. Tissue resident DCs are professional APCs and become activated when they recognise and phagocytose pathogens in infected tissue. Cytokines such as TNFα stimulate the migration of DCs to local lymph nodes where pathogen-derived antigens are processed and presented on the cell surface. However, the recognition of antigen-MHC class II complexes alone is not sufficient to activate T cells. In addition, activated APCs up-regulate expression of costimulatory molecules which provide essential signals for successful T cell activation. Activated DCs express large amounts of costimulatory molecules and as a consequence are potent stimulators of naïve T cells. Activation of naïve T cells is often referred to as "priming" and is an essential step in adaptive immunity.

T lymphocytes can be divided into two subsets according to the cluster of differentiation (CD) proteins on their cell surface. $CD4^+$ T cells recognise antigen-MHC class II complexes, whereas $CD8^+$ T cells recognise antigen-MHC class I complexes. Naïve T cells that encounter antigen in the correct format proliferate

(clonal expansion) into effector T cells ($CD8^+$ cytotoxic T cells and $CD4^+$ T helper (Th) cells) and memory T cells.

Unlike T cells, B cells can recognise antigen in its native state. The B cell receptor (BCR) is a cell membrane anchored immunoglobulin (Ig) of the same antigen specificity that will ultimately be secreted as antibody (Ab). There are five subclasses of Ab (IgA, IgD, IgE, IgG and IgM), which are each specialised in activating different effector mechanisms upon binding antigen. However, B cells cannot proliferate or become activated by antigen recognition alone and require help from antigen specific effector $CD4^+$ Th cells. This is achieved when B cells ingest antigen and present it in association with MHC class II molecules on their surface. $CD4^+$ Th cells, that express TCRs for the same antigen, bind the antigen-MHC class II molecules and deliver signals (co-stimulatory molecules and cytokines) that stimulate B cell clonal expansion. This results in the formation of Ab secreting plasma cells and memory cells.

Memory cells generated in this primary immune response enable a rapid secondary response to subsequent antigen exposure. This is because antigen specific cells are increased in comparison to when the antigen was first encountered. This secondary response produces a larger number of antigen specific effector T and B cells and in the case of B cells, induces greater levels of Ab secretion.

The generation of effector T cells constitutes cell-mediated immunity. Circulating effector T cells bind cell adhesion molecules via cell surface integrins and migrate to the site of infection. Once activated these T cells secrete cytokines and chemokines that increase the recruitment of immune cells and enhance inflammation.

Antigen specific cytotoxic $CD8^+$ T cells are responsible for eliminating virally infected cells. Antigen derived from intracellular viruses is presented on the surface

of infected cells in association with MHC class I molecules. This marks the cell as a target for cytotoxic $CD8^+$ T cells, which bind antigen-MHC class I complexes and kill the infected cell by inducing apoptosis.

Activation of antigen specific effector $CD4^+$ Th cells, via antigen-MHC class II recognition, stimulates the release of cytokines such as IL-2 and IL-4. These cytokines have autocrine effects that induce proliferation and differentiation. Until recently, $CD4^+$ Th cells were divided into two distinct subsets depending on the cytokine profile they secrete. Type 1 Th (Th1) cells secrete IL-2 and interferon gamma (IFNγ), whereas type 2 Th (Th2) cells secrete IL-4, transforming growth factor beta (TGFβ) and IL-10. These cytokines drive different immune responses. Th1 cells convey cell-mediated immunity (macrophage activation and T-cell-mediated cytotoxicity) and are associated particularly with obligate intracellular pathogens, whereas Th2 cells are associated with Ab responses and are important in host defence against parasitic infections. However, it is also recognised that Th1 cells stimulate production of specific antibody isotypes as well.

The production of large amounts of Ab from plasma cells facilitates the hosts defence by acting as an opsonin or by forming antigen-antibody complexes, which activate the classical pathway of complement. Immune cells such as neutrophils and macrophages then eliminate the invading pathogen by phagocytosis or cytotoxic mechanisms. Differentiation down either the Th1 or Th2 cell pathway is under the control of cytokines produced in response to the invading pathogen. For example, production of IL-12 in response to pathogen stimulates Th1 cell formation, whereas production of IL-4 stimulates Th2 cell formation. Whilst working on this thesis a further Th subset, termed "Th17", has been described. This Th subset will be discussed in more detail later on in the thesis.

Normally, innate and adaptive immune responses remove pathogens and inflammation subsides with normal function being restored. For a detailed review of immunity refer to Delves and Roitt *et al.* (2000).

1.3 Hypersensitivity

When immunological reactions cause more harm than good they are described as "hypersensitivity responses". These may occur when the body sees self-proteins as foreign (autoimmune responses) or where responses to otherwise innocuous antigens are inappropriate such as in allergy. Such responses may cause tissue injury and disease.

As in adaptive immunity, hypersensitivity responses are mediated by the generation of antigen specific Abs and antigen specific T cells. There are four types of hypersensitivity response. Types I-III are immediate type hypersensitivity responses that occur within minutes to hours, whereas type IV is a delayed type hypersensitivity (DTH) response that occurs 24-48 hours later. Type I is mediated by the production of IgE Abs which bind receptors on mast cells that recognise the fragment crystallisable (Fc) component of the Ab. Antigen binding to IgE causes activation of the mast cell which releases inflammatory mediators. This type of hypersensitivity is associated with allergy. Type II is mediated by IgG Abs that bind cell surface antigens. This type of hypersensitivity is associated with some drugs that bind to the surface of cells and act as a target for anti-drug IgG antibodies that cause destruction of the cell. Type III is again mediated by IgG antibodies, which bind soluble antigen and form antigen-IgG immune complexes that are deposited in tissue. These immune complexes induce complement activation and neutrophil recruitment resulting in a local inflammatory response. Type IV is mediated by antigen specific effector T

lymphocytes such as Th1 cells, which enter the site of exposure and recognise antigen-MHC class II molecules on APCs. These stimulated Th1 cells secrete chemokines and pro-inflammatory cytokines such as IFNγ which stimulate inflammation. Newly recruited macrophages present antigen which amplifies the response. The release of IFNγ and TNFα in this inflammatory response also activates macrophages which perpetuates the reaction. Each step in this type IV DTH response takes several hours, thus inflammation is delayed. For a review of hypersensitivity responses see Janeway and Travers. (1996).

1.4 Autoimmunity

The successful deployment of an appropriate immune response depends on the body being able to distinguish foreign-antigen from self-antigen (autoantigen). Mechanisms exist that prevent reactivity to autoantigens while allowing a response to foreign antigens. These mechanisms normally occur in the thymus and bone marrow during development and include deletion, receptor editing and anergy of potentially autoimmune lymphocytes. If auto-reactive clones survive these mechanisms they are suppressed by regulatory cells. However, these processes can fail and self-tolerance can be broken allowing self-reactive lymphocytes to mature. In these circumstances the autoantigen becomes the target of the immune response and leads to autoimmune disease. Systemic autoimmune diseases such as rheumatoid arthritis can affect multiple organs and are generally chronic because the autoantigen can never be cleared from the body. Autoimmune diseases can involve both the humoral and cellular components of immunity, with autoantibodies and self-reactive T cells driving the immune response against a specific autoantigen. The chronic inflammatory nature of these diseases usually results in tissue damage, which leads to

further release of autoantigen and increased recruitment of inflammatory immune cells that release more cytokines and chemokines. This results in a continuing and evolving self-destructive process that has severe consequences for affected individuals.

1.5 Rheumatoid arthritis

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease of the joint and is estimated to affect between 0.5 and 1% of the adult population worldwide (Kvien. 2004). Inflammation of the synovial tissue in the joint results in the development of a symmetrical polyarthritis with onset occurring usually between 40 and 50 years of age. However, juvenile forms of the disease exist and these can be particularly aggressive. Affected individuals present with pain, stiffness and swelling of the joints and may also suffer from fatigue, fever and weight loss. This has an impact on the patient's ability to perform routine daily tasks leading to reduced productivity at work and early retirement. RA is also associated with a reduced life expectancy of approximately 5 to 10 years. The direct costs of managing this disease as well as the indirect costs through loss of work represent a large economic burden.

1.5.1 RA aetiology

There is no linkage of the disease to socio-economic factors but women are over three times as likely to suffer from the disease as men. Genetic and environmental factors seem to play a role in the pathogenesis of RA. Serologically it was discovered that over 80% of patients with RA have a shared epitope (amino acids 70-74 of the major histocompatibility complex (MHC) class II beta chain) conserved in the Human Leukocyte Antigen (HLA) DR-1 and HLA-DR4 haplotypes (DRB*0101, DRB*0404

and DRB*1402) (Stastny. 1983). In addition, patients with severe disease are more likely to be HLA DR positive than patients with less aggressive RA. The HLA-D region encodes genes for MHC class II molecules, which bind to and present antigens to the TCR of $CD4^+$ T cells. However, not all RA patients are HLA DR positive and not all HLA DR positive individuals will develop RA suggesting non-inherited factors may also have a role to play in the aetiology of RA. Environmental triggers have therefore been implicated in disease initiation and include smoking as well as viral and bacterial infections.

Researchers have also assessed the usage of subfamilies of TCRs to try and identify the pathogenic T cells involved in RA. In some patients there appears to be a preferential usage of certain TCR beta chain variable regions on T cells from diseased joints (Mima *et al.*, 1999). This suggests, in patients that have a T cell driven disease, that there are dominant T cell subpopulations in the joint and that these cells may be driven by a shared antigen. These data suggest that T cells are important in the pathology of RA.

Considerable research has been conducted in an attempt to identify a specific autoantigen in RA. The identification of autoantibodies against collagen type II, chondrocyte glycoprotein 39, proteoglycans and citrullintaed proteins have suggested that these may be potential autoantigens in RA. However, their role in disease pathogenesis is not clear and the actual antigen responsible for the initiation of RA has not yet been identified.

Further attempts to understand the pathogenesis of RA have come from genome-wide single nucleotide polymorphism studies. These studies have identified genes associated with disease susceptibility. RA-associated polymorphisms have been described for a number of genes such as PADI4, a gene involved in the production of

peptidylarginine deiminase, which converts arginine to citrulline in proteins, and IL-23R, a cytokine receptor involved in IL-17 production (Yamamoto and Yamada. 2005 and Hollis-Moffatt *et al.*, 2008 respectively). Studies like these identify genetic risk factors in RA and lead to a better understanding of the biological pathways involved in disease, which may lead to novel therapeutic approaches.

In general RA is thought to occur when the immune system is inappropriately activated in susceptible individuals, probably through recognition of self-antigen and involves both the humoral and cellular components of immunity. In affected individuals the cells lining the joint become hyperplastic and mainly consist of activated macrophages (type A synoviocytes) with the underlying layer consisting of fibroblast-like cells (type B synoviocytes). Deeper layers within the synovium may have follicles of lymphoid cells around vessels as well as lymphocytes scattered between them. Macrophages and T lymphocytes represent the most abundant cell types in the synovial tissue with B cells, plasma cells, DCs and fibroblasts also being present. Refer to VanderBorght *et al.* (2001) for a review of RA aetiology and pathogenesis.

1.5.2 RA pathogenesis

Disease pathogenesis is thought to be initiated when the unknown pathogenic determinant is processed and presented by APCs to $CD4^+$ T cells. This interaction is mediated by binding of integrins such as lymphocyte function associated antigen-1 (LFA-1) and costimulatory molecules such as OX40, that are expressed on T cells, to ICAM-1 and OX40 ligand (OX40L) respectively on APCs. These associations result in activation of $CD4^+$ T cells. Both LFA-1 and OX40 have been shown to be

expressed on synovial fluid T cells in RA patients (Ueki *et al.*, 1994 and Giacomelli *et al.*, 2001 respectively).

In addition, $CD4^+$ T cells from RA patients express high levels of the costimulatory molecule CD40L on their cell surface (Toubi and Shoenfeld. 2004), which binds CD40 on B cells. This interaction stimulates B cells to produce immunoglobulin (Ig) such as rheumatoid factors (RFs). RFs are autoantibodies found in the majority of RA patients and titres of which are often used to indicate disease activity. Patients who are positive for RF are referred to as "sero positive". These autoantibodies bind the Fc component of circulating IgG antibodies and form immune complexes. The classical RF is an IgM antibody which binds IgG forming IgM-IgG complexes that are deposited in the joint. These complexes activate complement resulting in joint inflammation and may be responsible for disease perpetuation and/or the localisation of the inflammatory response to the joint.

RF has been detected early in RA, prior to clinical signs of disease, suggesting it may have a role to play in the initiation of disease. However, RA also occurs in individuals who are sero negative for RF suggesting T cells may drive the disease process in these patients.

In response to mediators of inflammation vascular endothelial cells increase their expression of adhesion molecules such as E-selectin and P-selectin, which facilitates cellular recruitment. Recruited neutrophils release elastase and other proteases that degrade proteoglycan in the superficial layer of cartilage. Depletion of proteoglycan enables immune complexes to precipitate in the superficial layer of collagens in the joint allowing inflammation to persist. Neutrophils also attract macrophages that secrete IL-1β which stimulates the increased expression of the adhesion molecules ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells.

These bind integrins such as LFA-1 and very late antigen 4 (VLA-4) respectively that are expressed on $CD4^+$ T cells. These cells then traffic to the inflamed synovial tissue. All these adhesion molecules and integrins have been detected in RA patients (Tak and Thurkow *et al.*, 1995 and Ueki *et al.*, 1994 respectively).

IL-1β is also present in the joints of RA patients (Schlaak *et al.*, 1996) and plasma levels have been shown to correlate with disease severity (Eastgate *et al.*, 1988), demonstrating its importance in disease pathogenesis.

$CD4^+$ T cells from the joints of RA patients produce IFNγ and IL-2 which are characteristic Th1 cell cytokines (Buchan *et al.*, 1988 and Miltenburg *et al.*, 1992), whereas they only produce low levels of characteristic Th2 cell cytokines such as IL-4 (Miossec *et al.*, 1990). This has lead to the hypothesis that RA is a Th1 cell driven disease. INFγ, as well as other cytokines, is able to activate macrophages leading to an increase in the transcription/production of TNFα, which is again elevated in RA (Schlaak *et al.*, 1996). TNFα and IFNγ act synergistically to increase the expression of adhesion molecules causing further cellular influx. Thus, it is the $CD4^+$ T cell that is thought to orchestrate this chronic inflammatory response. However, IFNγ levels in RA are relatively low and its significance in disease pathology is not clearly defined as it may also have anti-inflammatory properties (Ruschen *et al.*, 1989).

The inflammatory environment within the joint also stimulates macrophages and fibroblasts to produce other pro-inflammatory cytokines such as IL-6 and granulocyte macrophage colony-stimulating factor (GM-CSF). These have been detected in RA patients (Field *et al.*, 1991 and Haworth *et al.*, 1991 respectively) and have a plethora of biological actions. IL-1β and TNFα as well as aiding in cellular recruitment also have a broad range of other biological actions in RA and along with IL-6 and GM-CSF regulate the inflammatory response.

The acute phase response in RA is also activated by many of these pro-inflammatory cytokines and levels of CRP in the blood have been shown to correlate to disease severity (Larsen. 1988). Although the contribution of acute phase proteins to disease pathogenesis is unclear, their presence is often used as a marker of inflammation.

Given the degree of lymphocyte recruitment into the joint it is surprising that T cell derived cytokines such as IFNγ, IL-2 and IL-4 are not dramatically over-expressed. This has brought into debate the importance of $CD4^+$ T cells in RA. However, the production of IL-17, another T cell derived cytokine, has been shown to be abundant in RA (Chabaud *et al.*, 1999) suggesting these cells may have an important role in disease pathology. The expression of other cytokines such as IL-7 (Harada *et al.*, 1999), IL-12 (Schlaak *et al.*, 1996), IL-15 (McInnes *et al.*, 1996) and IL-18 (Gracie *et al.*, 1999) have been detected in RA and are capable of activating Th cells further supporting a role for T cells in disease.

At the same time as the pro-inflammatory response, anti-inflammatory mediators are also present in RA such as IL-4, IL-10 and transforming growth factor beta (TGFβ) (Schlaak *et al.*, 1996). In addition, IL-1 receptor antagonist (IL-1Ra, Firestein *et al.*, 1992) and soluble TNF receptors (sTNFR, Cope *et al.*, 1992) have also been detected. These molecules have been shown to inhibit inflammatory processes. However, there appears to be a loss of negative regulation in RA.

The B cell, as well as secreting autoantibodies, may also contribute to disease pathogenesis by producing cytokines such as IL-6. Cytokines derived from B cells promote leukocyte infiltration into the joint, formation of ectopic lymphoid structures, angiogenesis, and synovial hyperplasia. However, the precise cytokine pattern secreted by B cells in the synovium has yet to be determined. In addition, B cells are efficient antigen presenting cells and have been shown to mediate T cell activation in

RA (Takemura *et al.*, 2001). Thus, it would appear that both T and B cells are essential in developing an autoimmune response in the joint.

Chronic inflammation in the joint leads to cartilage destruction and bone erosion, which is a major cause of disability in RA patients. This occurs when the inflamed synovium forms an outgrowth known as the pannus that invades the cartilage and bone. The pannus is comprised of mesenchymal cells such as synovial fibroblasts, macrophages and lymphocytes.

Fibroblasts and activated T cells express the cytokine receptor activator of nuclear factor-κB ligand (RANKL), which is up-regulated in rheumatoid synovial tissue (Shigeyama *et al.*, 2000). RANKL binds its receptor RANK on osteoclast precursor cells that are recruited to the inflamed synovium. Osteoclast precursor cells then differentiate into mature osteoclasts that actively resorb bone. This process is regulated by osteoprotegerin (OPG), a soluble decoy receptor for RANKL, which is also expressed in synovial tissue of RA patients (Vanderborght *et al.*, 2004). OPG has been shown to inhibit osteoclast formation and activation (Simonet *et al.*, 1997). However, in RA there is an imbalance in the ratio of RANKL to OPG which favors RANKL induced osteoclastogenesis and bone resorption.

Other pro-inflammatory cytokines such as TNF, IL-1β, IL-6 and IL-17, that are abundant in the inflamed joint, can induce the expression of RANKL and enhance osteoclast driven bone erosion. Many of these cytokines also induce the release of matrix metalloproteinases (MMPs) such as stromelysin and collagenase from mesenchymal cells and osteoclasts. MMPs degrade connective tissue matrix and are important contributors to joint damage in RA (Vincenti *et al.*, 1994). In addition,

synovial cytokines are important mediators of angiogenesis in RA, which is required for sustained chronic inflammation and joint destruction.

Untreated, this chronic disease soon results in joint deformity with a severe reduction in the quality of life for the patient. This disease may also manifest as a systemic inflammatory condition at extra-articular sites giving rise to rheumatoid nodules, ocular disease, pulmonary fibrosis, vasculitis, pericarditis and myocarditis, which ultimately reduce life expectancy.

1.5.3 Diagnosis

RA is diagnosed using the American College of Rheumatology (ACR) classification criteria (Arnett *et al.*, 1988) as outlined in Table 1.1. Four or more of the criteria have to be met for at least four weeks before RA is diagnosed. This ACR system is useful in diagnosing active disease. However, in early RA where patients may present with single joint involvement and no radiographic damage a firm diagnosis cannot be made using this system (Saraux *et al.*, 2001). Joint erosions are present in 50-70% of patients two years after the onset of symptoms (Plant *et al.*, 1998) hence there is a need to diagnose RA as soon as possible. Earlier markers of RA are being developed and include assessment of antibodies to citrullinated peptides and better imaging techniques. If these prove to be accurate, treatment could be initiated earlier which may improve prognosis.

1.5.4 Clinical assessment of anti-arthritic drugs

In clinical trials the anti-arthritic effects of novel drugs are assessed using the ACR core data set of measures (Felson *et al.*, 1993), as outlined in Table 1.1. A drug is

assessed by its ability to improve the ACR score by 20, 50 or 70%, with these thresholds being referred to as ACR20, ACR50 and ACR70 respectively.

ACR diagnosis criteria	**ACR core data set for clinical trials**
Morning stiffness for at least one hour	Number of swollen joints
Soft tissue swelling of three or more joints	Tender joint count
Swelling (arthritis) of hand or wrist joints	Physicians assessment of global status
Symmetrical swelling of joints	Patients self-report including physical function, pain and global assessment
Rheumatoid nodules	Presence of one acute phase reactant, either erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP)
Presence of IgM RF in abnormal amounts	Radiographic assessment of hands and feet in clinical studies of 1 year duration or longer
Radiographic changes showing erosions and/or peri-articular osteopenia in hand and/or wrist joints	

Table 1.1 American College of Rheumatology diagnosis and clinical trial assessment criteria.

1.5.5 Current treatment

Despite the fact that 50% of patients are unable to work within five years of diagnosis, RA has until recently been regarded as something of a benign disease. This

has been reflected in the way the disease has been clinically managed. Most patients would be started on non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, progressing (as their disease progressed) to steroids such as prednisone and then to so called "disease modifying anti-rheumatic drugs" (DMARDs) such as methotrexate (MTX) and ending perhaps with joint replacement. The modern approach is to treat more aggressively from the start with DMARDs (Fries, 2000), partly because of the gastric side effects of NSAIDs and because NSAIDs provide only symptomatic relief and have no impact on disease progression. The most widely used of the DMARDs is MTX taken by approximately 50% of patients on DMARD therapy. Other commonly used DMARDs include hydroxychloraquine, leflunomide, sulfasalazine and azathioprine. MTX and leflunomide are effective in RA and have been shown to inhibit disease after 52 weeks of treatment with 46% and 52% of patients achieving an ACR20 respectively (Strand *et al.*, 1999). However, it is estimated that only 20-30% of patients remain on treatment after five years with most DMARDs and 40% of patients on MTX discontinue treatment over this time period (Simon, 2000). Cessation of treatment with these drugs is normally due to adverse events and/or loss of response when used for long periods of time. As a consequence patients often progress from one DMARD to another or use them in combination.

A recent breakthrough in the treatment of RA has been the targeting of the pro-inflammatory cytokine TNFα with biological therapies (biologics). Biologics are protein-based drugs derived from living organisms that target specific components of the immune system. The biologics currently used in the clinic that inhibit TNFα are etanercept (Enbrel), a fusion protein of the human TNF receptor-2 fused to a human IgG1 Fc; infliximab (Remicade), a chimeric monoclonal Ab (mAb); and adalimumab (Humira), a human mAb. These anti-TNFα drugs have all been shown to improve

symptoms and signs of disease in RA. In clinical practice anti-TNFα therapy is used to treat patients who have failed to respond to one or more DMARD. In these patients DMARD therapy is normally continued with anti-TNFα treatment. Studies have demonstrated that anti-TNFα treatment in patients receiving concomitant MTX inhibits disease more effectively than MTX alone (Table 1.2). In addition, anti-TNFα therapy has been shown to prevent progression of joint erosions in RA (Keystone *et al.*, 2004). These studies clearly show the benefit that anti-TNFα biologics have to offer in the treatment of RA.

Drug treatment	**Duration of treatment**	**ACR20 response**	**Reference**
Etanercept + MTX MTX only	24 weeks	71% 27%	Weinblatt *et al.*, 1999
Infliximab + MTX MTX only	30 weeks	58% 20%	Maini *et al.*, 1999
Adalimumab + MTX MTX only	24 weeks	63% 30%	Keystone *et al.*, 2004

Table 1.2 Effect of anti-TNFα biologics in RA patients receiving concomitant MTX.

In addition to these approved TNF inhibitors others are also in development such as certolizumab pegol (Cimzia), a pegylated fragment of antigen binding (Fab) fragment, which will give more choice to the physician and patient. However, 20-40% of patients are described as "non-responders" to anti-TNF therapy and these patients may benefit from other biological drug approaches.

Another available biologic is anakinra (Kineret), the IL-1 receptor antagonist (IL-1ra), which has been shown to be active in reducing disease symptoms in RA with

43% of patients achieving an ACR20 after 24 weeks of treatment (Bresnihan *et al.*, 1998) and is effective in combination with MTX (Cohen *et al.*, 2002). These results along with a number of other studies have shown IL-1 blockade to be effective in the treatment of RA. However, the effects seen with anakinra were modest compared to those of the TNF inhibitors. Other available biologics include rituximab (MabThera) and abatacept (Orencia). Rituximab is a depleting mAb directed against the CD20 antigen on B cells and abatacept is a fully human Fc fusion protein, comprising the extracellular portion of cytotoxic T-lymphocyte antigen 4 and the Fc fragment of human IgG1, which inhibits CD28 T cell costimulation. Both rituximab and abatacept have been shown to be effective in RA (Edwards *et al.*, 2004 and Kremer *et al.*, 2003 respectively).

The successful inhibition in the signs and symptoms of RA with the drugs outlined above have validated TNFα, IL-1, B cells and T cells as important components in RA pathogenesis.

1.5.6 Future treatments

Following the success of anti-TNF biologics other pro-inflammatory cytokines and their pathways are being targeted with biologics and small molecules. These novel drugs are outlined in Table 1.3. The most advanced of these emerging treatments is tocilizumab a humanised mAb against the IL-6 receptor. In a clinical trial 78% of RA patients achieved an ACR20 after 12 weeks administration of tocilizumab (Nishimoto *et al.*, 2004), which has validated a role for IL-6 in RA.

Target	Drug	Format	Development stage
IL-1β	Canakinumab	Anti-IL-1β Ab	Phase II
IL-6	Tocilizumab (Actemra)	Anti-IL-6 receptor Ab	Phase III
IL-12/IL-23	ABT-874	Anti-IL-12/IL-23 Ab	Phase III
IL-15	HuMax (AMG 714)	Anti-IL-15 Ab	Phase II
IL-17	AIN-457	Anti-IL-17 Ab	Phase II
IL-18	IL-18 binding protein	IL-18 antagonist	Phase II
BLyS	Belimumab (LymphoStat-B)	Anti-BLyS Ab	Phase II
BLyS and APRIL	Atacicept	Extracellular portion of TACI-human IgG1Fc fusion protein	Phase II
RANKL	Denosumab (AMG 162)	Anti-RANKL Ab	Phase II
CD20	Ocrelizumab	Anti-CD20 Ab	Phase III
p38	VX-702	p38 MAPK inhibitor	Phase II
JAK2	INCB-18424	JAK2 inhibitor	Phase II
CCR5	AZD-5672	CCR5 inhibitor	Phase II

BLyS, B lymphocyte stimulator; APRIL, a proliferation-inducing ligand; MAPK, mitogen-activated protein kinase; JAK, janus kinase.

Table 1.3. Drug targets and development stage of novel therapies.

1.5.7 IL-17 as a drug target

From the list of novel targets (Table 3) IL-17 has attracted a lot of interest in recent years. IL-17 is considered a T cell derived cytokine and has been detected in activated $CD4^+$ T cells (Yao *et al.*, 1995) and has also been shown to be produced by memory $CD4^+$ T cells (Aggarwal *et al.*, 2003). In RA, T cells resident in the synovial tissue have been demonstrated to produce IL-17, which is thought to contribute to inflammation (Chabaud *et al.*, 1999). In addition, pre-clinical animal models of RA

have shown that IL-17 deficiency and blockade inhibit signs of arthritis (Nakae *et al.*, 2003 and Lubberts *et al.*, 2001). Thus, these data have implicated a role for IL-17 in RA and suggest it is a promising target for drug intervention.

There are numerous therapies in development with the potential for treating RA patients. What is not clear is how to differentiate between these new therapies in terms of identifying the advantages one has over another for a given patient population without conducting lengthy and expensive clinical trials.

1.6 Collagen-induced arthritis

In drug development animal models are used to identify and validate novel therapies which may ultimately be used in the clinic to treat patients. In non-clinical pharmacology the most favoured model of RA is collagen-induced arthritis (CIA), which is an inflammatory polyarthritis model. The development of arthritis after sensitisation to heterologous collagen type II (CII) in complete Freund's adjuvant (CFA) was first reported by Trentham *et al.* in 1977 in the rat. In 1980 Courtenay *et al.* reported similar findings in the mouse. CFA, which contains mycobacterium tuberculosis, is essential to elicit CIA demonstrating that TLR stimulation is required to initiate an immune response. Since these initial studies the specific CII arthritogenic epitopes have been identified within peptide fragments $CII_{181\text{-}209}$ and $CII_{245\text{-}270}$ (Myers *et al.*, 1993) suggesting preservation of these epitopes is required. However, intact CII, which is processed and presented on the surface of APCs to T cells, is still routinely used to elicit disease. CII presentation by APCs initiates cell contact and stimulates molecular processes such as the increased expression of

costimulatory molecules on the cell surface which is essential for the initiation of an immune response.

CIA is a long term model and not all animals will develop disease. It is also sensitive to other factors such as stress which may affect disease development. In addition, it has been observed that animals acclimatised for a longer period of time prior to the initiation of CIA develop a more robust disease (Unpublished observation). Environmental conditions therefore appear to play a role in disease manifestation but the factors involved are unclear. Despite these issues, CIA is still routinely used in drug discovery as it shows many similarities to RA. However, it is a model and also differs from RA in a number of respects. The similarities and differences between CIA and RA, as reviewed by Trentham (1982), are summarised in Table 1.4. All commonly used anti-arthritic therapies are effective in CIA models (Bendele *et al.*, 1999) including biological agents directed against TNFα (Piguet *et al.*, 1992) therefore demonstrating that CIA is a clinically relevant model of RA.

Similarities to RA	Differences to RA
Susceptibility linked to MHC	Disease is induced
Immunologically mediated	The model "resolves"
Erosions of cartilage and bone	New bone formation more marked
Autoimmunity to collagen seen in some RA patients	No synovial vasculitis
Disease centered on joints	No cycles of relapse and remission
	No RF
	No sex predilection
	CIA is acute RA is chronic

Table 1.4 Similarities and differences of CIA to RA.

CIA is normally conducted in DBA/1 mice. This strain of mouse is genetically susceptible to CIA as it is H2q restricted and presents CII on an MHC class II background to $CD4^+$ T cells. Generation of CII specific T cells enables the proliferation of B cells and production of anti-CII antibodies (Hom *et al.*, 1986). Antibodies against CII are also detected in up to 70% of patients with RA (Cook *et al.*, 1996). Thus, like RA, CIA involves both the humoral and cellular components of the immune system. This is highlighted by the fact that mice deficient in $CD4^+$ T cells (Taneja *et al.*, 2002) or B cells (Svensson *et al.*, 1998) are resistant to CIA. Adoptive transfer studies have shown that T cells alone are not sufficient to transfer severe disease into recipient mice and only mild transient symptoms are seen (Kakimoto *et al.*, 1988). Likewise, antibody transfer alone from immunised donors causes only mild arthritis in recipients (Stuart & Dixon, 1983). However, Seki *et al.* (1988) demonstrated that transfer of T cells and serum to naïve mice resulted in severe and

sustained arthritis. In addition, Tada *et al.* (1999) showed that the transfer of T and B cells resulted in the development of arthritis.

These data illustrate that both the humoral and cellular components of the immune system are essential in the elicitation of CIA. Further support for the role of B cells in CIA has recently been provided by Holmdahl *et al.* (2002) who showed that B cells are capable of processing and presenting CII to primed T cells. This study also showed that primed B cells activated T cells two-three times more effectively than naïve B cells, suggesting B cells have the potential to play an important role as APCs in the immune response to CII. In RA, cellular responses to CII have also been demonstrated. Trentham *et al.* (1978) showed that cells isolated from RA patients are activated by CII and Kim *et al.* (1999) showed that T cells proliferate in response to CII stimulation, therefore suggesting CII may have a role to play in the pathogenesis of RA.

The presence of $CD4^+$ T cell subsets in CIA has been investigated and Mauri *et al.* (1996) demonstrated that cells isolated from lymph nodes, six days post-CII sensitisation, produce IFNγ. Re-stimulation of these cells with CII resulted in an approximate ten-fold increase in IFNγ production suggesting activation of an antigen specific Th1 cell response. At the same time point, Th2 cell cytokine production was suppressed. However, during progression and subsequent resolution of arthritis IL-10 levels increased and low levels of IL-4 were present throughout the time course. This suggests that Th2 cell responses may have a role to play in CIA but probably during resolution and may suppress arthritis. This study also showed that during the progression and resolution of disease IFNγ levels decreased, therefore indicating that Th1 cell responses are dominant early in the immune response to CII and may cause the suppression of Th2 cell responses with the reverse being true later on in the

disease course. Similar data was also obtained by Doncarli *et al.* (1997) who showed a dominant IFNγ secreting Th1 cell population 15 days post-sensitisation with an increase in IL-4 secreting Th2 cells on Day 30. These data have led to the theory that CIA, like RA, is a Th1 cell driven disease. However, there is controversy surrounding the role of IFNγ in disease pathogenesis. Some researchers suggest IFNγ exacerbates disease (Cooper *et al.*, 1988 and Boissier *et al.*, 1995) and others suggest it protects against disease (Nakajima *et al.*, 1990 and Vermeire *et al.*, 1997). These studies have brought into doubt the importance of $CD4^+$ Th1 cells in CIA.

The immune response in pre-clinical models of arthritis, in terms of cellular interactions, cytokine and chemokine profiles, is similar to that in RA. OX40, CD40L (Saijo *et al.*, 2002), LFA-1, ICAM, VLA-4, VCAM, P-selectin, IFNγ, IL-4 (Hersmann *et al.*, 1998), IL-2, IL-1ra, TGFβ (Thornton *et al.*, 1999), IL-10 (Mauri *et al.*, 1996), IL-1β, TNFα, and IL-6 (Marinova-Mutafchieva *et al.*, 1997) are detected in the joints or produced by cells from arthritic animals. In addition, evidence from knockout mice or blockade of cytokine function has shown the importance of IL-12 (Murphy *et al.*, 2003), IL-15 (Ferrari-Lacraz *et al.*, 2004), IL-17 (Nakae *et al.*, 2003) and IL-18 (Banda *et al.*, 2003) in CIA. Chronically inflamed joints of CIA mice also show marked cartilage erosion and bone destruction similar to that seen in RA. Examination of synovial tissue from arthritic mice has identified the expression of RANK and RANKL which, like RA, are thought to play a key role in bone erosion (Lubberts *et al.*, 2002). This list of immunological similarities is by no means exhaustive.

CIA is, however, time-consuming with experiments typically lasting 50-70 days. The development of arthritis is not always well synchronised and not all animals develop

disease (Caccese *et al.*, 1992). This variability has consequences in terms of more animals being required for study. It also makes prophylactic dosing studies especially challenging because it is not a simple matter to differentiate between an animal that has responded to drug treatment and an animal that is a "non responder".

Prophylactic drug administration will take into account both the potential immunomodulatory and anti-inflammatory effects a drug may have in the CIA model. However, prophylactic dosing cannot distinguish between these effects. Studying drug effects therapeutically in animals with clinical signs of disease assesses a drugs anti-arthritic potential. Whilst many might argue that only therapeutic studies have relevance to human disease, the reality is that many immunomodulatory drugs, that have utility in the treatment of RA, are only active in CIA when dosed prophylactically. This suggests that the immunomodulatory activity of a drug is likely to contribute to its anti-arthritic effect. In addition, drugs that are effective therapeutically in CIA are usually also effective when administered prophylactically. It would therefore appear that studying the immunomodulatory activity of a drug gives an indirect assessment of its potential anti-arthritic action. For these reasons the prophylactic dosing regime is often favoured for assessing new drug entities.

CIA is usually assessed in terms of clinical scores (based on number and severity of swollen joints) and the degree of joint destruction as assessed by x-ray and or histopathology. In this model there is a relatively straightforward relationship between severity of clinical scores and underlying joint destruction (a relationship that is not so clear cut in RA). So drugs that are effective in CIA, whatever their mechanism of action, appear to have very similar profiles. The model is not therefore very good at differentiating between anti-arthritic drugs.

Ideally, the CIA model would be short, highly reproducible and have a variety of readouts that allow drugs to be differentiated on mode of action. To address some of the issues relating to CIA, an approach may be to develop short-term models that concentrate on the development of CII immunity in sensitised animals rather than waiting for arthritis development. Careful choice of readouts may then allow drugs to be differentiated mechanistically. Assessing drug effects in short-term models of CII immunity would evaluate their immunomodulatory activity and give an indirect readout of their anti-arthritic potential. Furthermore, if these models were robust and appeared predictive of drug effects in CIA, they would have the potential to reduce numbers of animals required for drug testing and the numbers of animals that needed to be rendered arthritic.

1.7 Hypothesis

The hypothesis to be tested is that short-term models of collagen II immunity will be predictive of drug effects in CIA and provide a series of readouts that will allow anti-arthritic drugs to be differentiated based on mechanism of action.

1.8 Early readouts of CII immunity

1.8.1 Anti-collagen II antibodies

In CIA, anti-CII antibodies are produced and are easily measured in serum using enzyme-linked immunosorbent assays (ELISA) giving a readout of the humoral immune status of the animal. Analysis of the different antibody isotypes produced in response to CII may also inform on the subset of $CD4^+$ Th cells that have been activated. IL-4 secreting Th2 cells have been demonstrated to support IgG1

production from plasma cells, whereas IFNγ secreting Th1 cells support IgG2a production (Stevens *et al.*, 1988). Thus, the presence of IgG2a and IgG1 are indicators of Th1 and Th2 cell responses respectively. Anti-collagen II IgG1 and IgG2a Abs are readily detected in the sera of CIA mice and Doncarli *et al.* (1997) showed that although these Abs are not seen eight days after sensitisation they are present on Day 25 and continue to increase over the course of disease. Also, a study by Williams *et al.* (1998) demonstrated that anti-heterologous and anti-homologous CII IgG Ab levels, detected in pre-arthritic mice 21 days post-CII sensitisation, showed a statistically significant correlation to subsequent disease severity. These studies demonstrate that CII specific Abs are produced in the pre-arthritic phase and suggest they are predictive of arthritis in CIA.

1.8.2 Serum amyloid A (SAA)

Levels of CRP in clinical trails are monitored in RA patients and anti-arthritic drugs have been shown to reduce levels (Weinblatt *et al.*, 1999), therefore indicating it is a valuable marker of drug efficacy. In the mouse, the acute-phase protein SAA is more responsive to inflammation than CRP. SAA levels can be easily detected in serum by ELISA and are present in arthritic mice (Palmer *et al.*, 2003). Thus SAA may be a potential marker of drug efficacy in the CIA model. However, it is not known if SAA can be detected in CII sensitised mice in the pre-arthritic phase.

1.8.3 Collagen II Hypersensitivity

Sensitisation to CII and subsequent re-challenge has been demonstrated to elicit hypersensitivity responses. Farmer *et al.* (1986) showed that a DTH response could be detected to CII in the ear four days after sensitisation and Seki *et al.* (1988)

demonstrated the same response in the footpad 14 days after sensitisation. In another study, Omata *et al.* (1997) showed that a DTH response, in the ear, could be elicited in mice with CIA 49 days after sensitisation. These data illustrate that animals sensitised and then re-challenged with CII, prior to disease onset, react in the same way as those with arthritis. This suggests that a CII-induced DTH response has the potential to be utilised as an early readout of CII immunity. Omata *et al.* (1997) also demonstrated that the clinically used DMARD, MTX, inhibits CIA and CII-induced DTH therefore suggesting this readout is responsive to anti-arthritic therapies. However, the effect of MTX was not assessed in the pre-arthritic phase and it is not clear if a CII-induced DTH response in this phase would predict drug efficacy in CIA. DTH responses are T cell mediated and can therefore be used to assess the effect of therapies on cell mediated immunity. Furthermore, it has been demonstrated that immediate type hypersensitivity responses often precede DTH responses (Titus and Chiller., 1981). If this is true of a CII-induced response then measurements taken a few hours post-re-challenge could give a readout of humoral immunity. Taken together these readouts may give useful information on drug mechanism of action.

1.8.4 Collagen II stimulated cell proliferation

Cellular immunity to CII is essential for the development of CIA. Cells isolated from the lymph nodes of CII sensitised mice have been shown to proliferate in response to CII *ex vivo* (Farmer *et al.*, 1986). This proliferative response is easily quantified by the addition of radioactive tritiated thymidine (^{3}H) to the culture which then becomes incorporated into dividing cells and is measured on a beta counter. Mauri *et al.* (1996) demonstrated that a statistically significant increase in proliferation of lymph node cells to CII could be detected in pre-arthritic mice 13 days post-sensitisation. This

cellular response therefore has the potential to be used as an early readout of CII immunity.

Most of these readouts of CII immunity appear to be detectable in pre-arthritic animals. However, they have, as yet, not been utilised to predict the effects of anti-arthritic drugs prior to disease onset. These models and assays need to be set up to assess the conditions needed to obtain robust and reproducible responses.

1.9 Pharmacological tools

To test the hypothesis set out in this thesis a variety of experimental, established and novel drugs were selected for assessment in CIA and short-term mechanistic models of CII immunity.

1.9.1 T lymphocytes in CII immunity

Anti-CD4$^+$ and anti-CD8$^+$ mAbs

The importance of CD4$^+$ T cells in the CIA model is well-established. It has been demonstrated that mice deficient in CD4$^+$ T cells are resistant or show reduced susceptibility to disease (Taneja *et al.*, 2002 and Ehinger *et al.*, 2001). Depleting or blocking mAbs against CD4 have also been shown to inhibit arthritis in mice (Ranges *et al.*, 1985 and Chu and Londei. 1996). In addition, anti-CD4 therapy has been shown to be active in RA patients (Tak *et al.*, 1995 and Choy *et al.*, 2000). Although this effect was only moderate, it does suggest that these cells have a role to play in established disease. Regardless of the therapeutic merit of depleting CD4$^+$ T cells in RA these cells represent an important target when trying to understand the cellular immune response to CII.

The role of $CD8^+$ T cells in CIA is more contradictory. Some researchers report that these cells are regulatory in CIA (Taneja *et al.*, 2002) whereas others report that mice deficient in $CD8^+$ T cells have a normal CIA response (Ehinger *et al.*, 2001). Thus, the contribution of $CD8^+$ T cells in disease pathogenesis is not fully understood. Depleting mAbs against $CD4^+$ and $CD8^+$ T cells will be used to confirm the role of these cells in CIA and to determine the underlying T cell population that drives the short-term models of CII immunity.

1.9.2 Immunomodulation of CII immunity

Anti-LFA-1, anti-CD40L mAbs and leflunomide

The T cell-APC and T cell-B cell interactions are fundamental in the initiation of adaptive immunity. These cellular associations are dependent on costimulatory molecules and cell adhesion molecules binding their ligands. T cell-APC contact and hence antigen recognition is dependent on the binding of LFA-1, expressed on T cells, to ICAM-1 expressed on APCs. The importance of this interaction on T cell activation has been demonstrated using Abs directed against LFA-1 which inhibit T cell proliferation (Dongworth *et al.*, 1985 and Berzins *et al.*, 1988). In addition, Kikimoto *et al.* (1992) showed that mAbs against LFA-1 and ICAM-1 suppress CIA. These studies demonstrate a role for LFA-1 in T cell activation and disease.

Antibody generation is important in the pathogenesis of RA and is mediated in part through the binding of CD40L on T cells to CD40 on B cells. In the CIA model it has been demonstrated that a mAb against CD40L (anti-gp39) prevents arthritis development and anti-CII Ab production (Durie *et al.*, 1993). These data indicate the importance of T cell–B cell contact and the subsequent humoral response in CIA.

Given the role of LFA-1 and CD40L in the initiation of an immune response, mAbs against these targets should have profound effects on early readouts of CII immunity. The use of mAbs that target these molecules will help underpin the relative contribution of T cell–APC and T cell–B cell interactions in CII immunity.

In addition to these experimental drugs, leflunomide, a DMARD used to treat RA, will also be utilised. The primary mechanism of action of leflunomide is the inhibition of *de novo* pyrimidine synthesis which is required for cell division (Ruckemann *et al.*, 1998 and Breedveld and Dayer. 2000). Lymphocytes have low cellular pools of pyrimidine and are therefore sensitive to leflunomide. Assessing the effect of leflunomide on pre-arthritic readouts will further highlight the role of lymphocytes in CII immunity. Leflunomide has been shown to be effective in experimental models of arthritis (Thoss *et al.*, 1996 and Schorlemmer and Schleyerbach. 1998) therefore demonstrating the relevance of pre-clinical animal models in anti-arthritic drug discovery. In this thesis leflunomide will be used to validate the murine CIA model and the pre-arthritic readouts of CII immunity.

1.9.3 Pro-inflammatory cytokines in CII immunity

Anti-TNFα and anti-IL-1β mAbs

The pro-inflammatory cytokines TNFα and IL-1β have a wide range of biological actions. Both of these cytokines increase ICAM-1 expression and synergise with each other to induce cellular infiltration to sites of inflammation (Doukas and Pober. 1990 and Wankowicz *et al*, 1988). These cytokines also regulate each others production (Brennan *et al.*, 1989 and Williams *et al.*, 2000) and are both capable of inducing other inflammatory cytokines such as PGE_2 and IL-6 secretion (Jorgensen *et al.*,

1991, Choy *et al.*, 1999 and Saijo *et al.*, 2002). Increased production of these cytokines therefore perpetuates the inflammatory response.

In the murine CIA model, mAbs directed against TNFα and IL-1β have been shown to inhibit the development of arthritis (Williams *et al.*, 1992 and Geiger *et al.*, 1993 respectively) therefore demonstrating the importance of these cytokines in disease. In addition, TNFα has been shown to stimulate bone resorption and inhibit bone formation *in vitro* (Bertolini *et al.*, 1986) and in the clinic anti-TNFα therapy has been shown to halt joint erosion in RA patients. Additional biological actions of IL-1β include induction of IL-2 production and augmentation of T cell proliferation (Hackett *et al.*, 1988).

It would appear that anti-TNFα therapy exerts its anti-arthritic effects by reducing inflammation and preventing bone erosion. In pre-arthritic animals, where the immunomodulatory properties of drugs are assessed, there is no evidence of inflammation or bone erosion so it is possible that the anti-arthritic effect of an anti-TNFα mAb will not be detected. However, the role of TNFα in the early stages of an immune response to CII has not been investigated. IL-1β also seems to play a predominantly pro-inflammatory role which may not be detected in the early readouts of CII immunity. However, its effect on T cell proliferation may be important in CII immunity and assessing an anti-IL-1β mAb in the pre-arthritic phase should establish this.

Anti-IL-6 mAb

Another pivotal cytokine in the immune response is IL-6, which has pleotropic actions. IL-6 is involved in the production of Ig from B cells (Croft and Swain, 1991), B cell differentiation (Splawshi *et al.*, 1990), T cell proliferation and IL-2 expression

(Pankewycz *et al.*, 1990), which are all essential in immune responses. Thus, it is not surprising that blockade of the IL-6 receptor (IL-6R) with a mAb inhibits CIA with a corresponding decrease in anti-CII antibody production (Takagi *et al.*, 1998). From these data the effect of a mAb directed against IL-6 should be detected in the pre-arthritic readouts, as it is likely to play an essential role in CII immunity.

Taken together, the use of these mAbs will identify the role of TNFα, IL-1β and IL-6 in CII immunity and may shed light on the mechanisms of action of drugs that target these cytokines in CIA.

The drugs outlined above will be used to characterise and validate the CIA model and the short-term readouts of CII immunity. Assessing such a broad range of drugs will determine if the pre-arthritic models and assays are predictive of drug effects in CIA. In addition, they should identify the key immunological processes involved in an immune response towards CII and it should become evident if these models and assays are capable of discriminating between drugs with different modes of action.

1.10 Evaluation of a novel drug in CII immunity

Anti-IL-17 mAb

Having characterised the pre-arthritic models and assays they will be utilised to assess the role of a novel drug target in CII immunity. Recently, IL-17 has been identified as another key pro-inflammatory cytokine and has been shown to induce the production of IL-1β, TNFα and IL-6 from macrophages (Jovanovic *et al.*, 1998). It is also able to synergise with IL-1β to enhance IL-6 production (Chabaud *et al.*, 1998) and with TNFα to enhance both IL-6 and IL-1β production from synoviocytes (Katz *et al.*,

2001). In addition, IL-17 has been demonstrated to stimulate the secretion of other inflammatory mediators such as IL-8 and granulocyte-colony-stimulating factor (G-CSF) which are involved in neutrophil recruitment (Fossiez *et al.*, 1996) and is also capable of inducing ICAM-1 expression (Yao *et al.*, 1995). Mice deficient in IL-17 are resistant to CIA (Nakae *et al.*, 2003) and blocking IL-17 has also been shown to reduce signs of arthritis (Lubberts *et al.*, 2001). Thus, IL-17 has become a focus for therapeutic intervention in recent years. However, its role in disease pathogenesis both in RA and CIA has not been fully established. Thus, IL-17 represents a novel target and the use of a mAb directed against this cytokine will be assessed in the CIA model and the pre-arthritic models and assays. This analysis may help to identify the role of IL-17 in arthritis and inform on the mechanism of action of this novel drug.

1.11 Aims

1. To assess drugs with varying mechanisms of action in the CIA model.
2. To develop short-term models of CII immunity in pre-arthritic mice.
3. To examine the effects of drugs utilised in aim 1 on short-term models of CII immunity and determine if these models are predictive of drug effects in CIA and whether they are capable of discriminating between drug mechanisms of action.
4. To investigate the role of the novel pro-inflammatory cytokine, IL-17, in CII immunity.

Chapter two

Materials and methods

2.1 Materials

2.1.1 Laboratory equipment

HEPA class 1000 incubator, Centra GP8R centrifuge used for cell harvesting, Multidkan EX microtitre plate reader, Wellwash 4 MK2 automated plate washer (Thermo Fisher Scientific, Loughborough, U.K.).

Eppendorf 5415D bench top microcentrifuge used for small-scale centrifugation (Anachem Ltd, Luton, U.K.).

Skatron cell harvester (Skatron Instruments Inc, Lier, Norway).

1205 betaplate liquid scintillation beta (β) counter (PerkinElmer, Beaconsfield, U.K.).

AID ELISpot plate reader and ELISpot reader version 4 software (AID ELISpot, Strassberg, Germany).

FACScalibur flow cytometer equipped with CellQuest software (BD Biosciences, San Jose, CA).

Callipers 0-25mm range (Miltutoyo Corporation, Kanagawa, Japan).

2.1.2 Commonly used materials

General lab consumables were freely available and purchased by UCB.

Chicken sternal type II collagen (MD Biosciences, Zurich, Switzerland).

Gibco sterile tissue culture grade phosphate buffered saline (PBS) (Invitrogen, Life Technologies, Paisley, Scotland).

Bovine serum albumin (BSA) (Sigma, Poole, U.K.).

Sulphuric acid (BDH Ltd, Poole, U.K.).

Nunc^{TM} ELISA plates supplied by Fisher Scientific (Loughborough, U.K.).

2.1.3 Materials specific to methods

CII sensitisation and CIA

Acetic acid (BDH Ltd, Poole, U.K.).

Complete Freund's adjuvant and incomplete Freund's adjuvant (Sigma, Poole, U.K.).

Anti-CII antibody ELISA

Phosphate buffered saline (PBS) powder concentrate (Fisher Scientific, Loughborough, U.K.).

Anti-mouse IgG1 and IgG2a - horseradish peroxidase conjugated (HRP) (Serotec, Kidlington, U.K.).

3,3',5,5' tetramethylbenzidine (TMB) (Sigma, Poole, U.K.).

Serum amyloid A ELISA

Serum Amyloid A (SAA) detection kit (Biognosis, Tridelta Development Ltd, Maynooth, Ireland) containing SAA antibody coated wells, suitable wash buffer concentrate, appropriate sample/calibrator diluent buffer concentrate, SAA standard calibrator (0.95μg/mL), anti-SAA-HRP conjugated antibody, TMB and stop reagent.

$CD4^+$ T cell isolation and cultures

Gibco RPMI 1640 medium, L-glutamine and penicillin-streptomycin (Invitrogen Life Technologies, Paisley, Scotland).

Heat inactivated foetal calf serum (FCS), Trypan blue solution, 2-mercaptoethanol. HEPES buffer, ethylenediamine tetra-acetic acid (EDTA), Red blood cell (RBC) lysis buffer, Mitomycin C (Sigma, Poole, U.K.).

Magnetic cell sorting (MACS) LS^+ columns and $CD4^+$ MACS separation kit containing biotin-antibody cocktail and anti-biotin microbeads (Miltenyi Biotec, Bisley, U.K.).

Methyl-[3H]-thymidine, specific activity of 50μCi (Amersham Biosciences, Chalfont St.Giles, U.K.).

Cytokine ELISA

Mouse IL-4, IL-17 and IFNγ DuoSet® ELISA development system kits, containing capture antibodies (rat anti-mouse IL-4, IL-17 and IFNγ), detection antibodies (biotinylated goat anti-mouse IL-4, IL-17 and IFNγ), standards (recombinant mouse IL-4, IL-17 and IFNγ) and streptavidin conjugated to HRP.

Substrate solution pack containing colour reagent A (hydrogen peroxide) and colour reagent B (TMB).

All supplied by R&D Systems Europe (Abingdon, U.K.).

Tween 20® (Sigma, Poole, U.K.).

Cytokine enzyme-linked immunospot (ELISpot)

Mouse IL-4, IL-17 and IFNγ ELISpot assay kits, containing mouse IL-4, IL-17 and IFNγ mAb coated 96 well microplates, detection antibodies (biotinylated polyclonal antibodies specific for IL-4, IL-17 and IFNγ), streptavidin alkaline phosphatase, suitable dilution buffers, suitable wash buffer, 5-Bromo-4-Chloro-3' Indolylphosphate p-Toluidine Salt (BCIP) and Nitro Blue Tetrazolium Chloride (NBT), and recombinant mouse IL-4, IL-17 and IFNγ positive controls.

Recombinant mouse IL-4, IL-17 and IFNγ.

All supplied by R&D Systems Europe (Abingdon, U.K.).

Flow cytometric analysis

Fluorescein isothiocyanate (FITC) conjugated rat anti-mouse IgG2b CD4 antibody (clone GK1.5), phycoerythrin (PE) conjugated rat anti-mouse IgG2a CD8 antibody (clone 53-6.7) and polypropylene Falcon tubes (BD Biosciences, Oxford, U.K.). Sodium azide (Sigma, Poole, U.K.).

2.1.4 Buffers, solutions and media

PBS wash buffer for ELISA plates	137mM NaCl, 2.68mM KCl, 10.1mM Na_2HPO_4, 1.76mM KH_2PO_4, pH 7.4
MACS buffer	Sterile tissue culture grade PBS (pH 7.2) supplemented with 0.5% bovine serum albumin (BSA) and 2mM EDTA. Sterile filtered and degassed.
Fluorescence activated cell sorter (FACS) buffer	Sterile tissue culture grade PBS (pH 7.2) supplemented with 5% FCS and 0.1% sodium azide
Cell culture media	RPMI 1640 medium containing 10% FCS, 1% penicillin-streptomycin, 0.2% 2-mercaptoethanol ($2x10^{-5}$M), 1% L-glutamine and 2.7% HEPES buffer.
RBC lysis buffer	Sterile tissue culture grade distilled water supplemented with 155mM NH_4Cl, 10mM $KHCO_3$ and 0.1mM EDTA, sterile filtered
Block buffer for ELISA	1% BSA in PBS

Reagent diluent for cytokine ELISA	0.1% BSA, 0.05% Tween 20 in Tris-buffered saline (20mM Trizma base, 150mM NaCl) pH 7.2, 0.2μm filtered
Stop solution for ELISA	2 N H_2SO_4

2.1.5 Drugs

Monoclonal antibodies were derived in-house and are outlined in Table 2.1 below.

Antibody	Construct	Affinity
Anti-CD4$^+$ (YTS 191)	Rat IgG2b	59nM
Anti-CD8$^+$ (YTS 169)	Rat IgG2b	72nM
Anti-CD40L (MR1)	Chimeric hamster IgG1	0.54nM
Anti-LFA-1 (Tib 217)	Rat IgG2a	Not known
Anti-TNFα (cTN3)	Murinised pegylated Fab	1.20nM
Anti-IL-1β (B122)	Hamster IgG1	0.42nM
Anti-IL-6 (54E07)	Murinised rabbit IgG1	0.02nM
Anti-IL-17 (Ab13)	Chimeric rabbit IgG1	0.03nM
Anti-IFNγ (H22)	Hamster IgG1	Not known

Table 2.1 Monoclonal antibodies

Monoclonal antibodies were formulated in either PBS (pH 7.4) or 50mM sodium acetate and 125mM sodium chloride (pH 5.0). All monoclonal antibodies were greater than 98% pure and endotoxin levels were less than 1 EU/mg. Prior to *in vivo* testing the monoclonal antibodies were characterised in appropriate *in vitro* cell based assays. Affinities were assessed either by FACS analysis or by a surface resonance technique known as "biacore". The affinity of anti-LFA-1 and anti-IFNγ are not

known. However, in an *in vitro* cell based assay anti-LFA-1 has been shown to block LFA-1 at 10μg/ml. In addition, anti-IFNγ has been demonstrated to block the antiviral activity of IFNγ at ng/mL concentrations, therefore indicating it is a potent inhibitor of IFNγ (Schreiber *et al.*, 1985). Leflunomide was utilised together with cyclosporin A (Sigma, Poole, U.K.).

2.2 Animals

Eight to ten week old male DBA/1 mice were purchased from Harlan (Oxfordshire, U.K.). Mice were housed in cages in an environmentally controlled room (temperature 21 °C to 23 °C and relative humidity 38% to 50%) on a 12-hour light/dark cycle according to U.K. Home Office regulations. Animals had access to RM1 food pellets (Lillico, Betchworth, U.K.) and water *ad libitum.* Animals were acclimatised for at least four weeks before use.

2.3 *In vivo* drug treatment

Drugs were administered prophylactically from one day prior to sensitisation (Day-1). The only exceptions were anti-$CD4^+$ and anti-$CD8^+$ mAbs, which were dosed 3 days prior to sensitisation. All control mice received a suitable vehicle. The dose and frequency of administration for each drug are stated in the results chapters. All drugs were dosed subcutaneously with the exception of leflunomide which was dosed orally.

2.4 Methods

2.4.1 Collagen-induced arthritis (CIA)

Induction of arthritis in mice requires sensitisation to CII and was originally described by Courtenay *et al.* in 1980. CII sensitised mice mount an immune response that manifests as an inflammatory polyarthritis in the paws. Arthritis is assessed visually as a clinical score. In this thesis, a protocol similar to that outlined by Courtenay *et al.* was used to induce arthritis in DBA/1 mice.

CII sensitisation

Chicken type II collagen (CII, isolated from chick sternal cartilage) was dissolved in 0.1M acetic acid at 2mg/mL and constantly rolled overnight in the dark at 4°C. The next day (Day 0) the CII solution was mixed with an equal volume of Complete Freund's Adjuvant (CFA), which contains 1mg/mL heat killed mycobacterium tuberculosis, to give a 1mg/mL solution. The solution was mixed with a syringe by repeatedly drawing up and down to produce an emulsion. The correct consistency was achieved when a drop of emulsion could be placed in water without sinking or dissipating. DBA/1 mice were injected with 0.1mL (100μg CII) of emulsion into the base of the tail, by an intradermal route under anaesthesia, until a white blister appeared just under the surface of the skin. Where indicated, normal mice (not sensitised) and mice sensitised with 0.1mL of emulsified CFA only were also used. Each mouse was checked 30 minutes after this procedure.

CII boost injection

On Day 14 post-sensitisation mice were given a booster injection of CII, following the same method as described in the CII sensitisation section, but with Incomplete Freund's Adjuvant (IFA) (containing no mycobacterium), instead of CFA.

From Day 14 onwards animals were checked daily for signs of arthritis. When signs were seen, animals were weighed, disease scored on a 0-3 scale per paw (as outlined below) and distress scored daily. Clinical score is the sum of all 4 paws (maximum score is 12 per mouse). Experiments were stopped once a robust disease was established in CII/CFA sensitised mice. Disease severity in the CIA model varies between studies. A robust disease response was considered to be one which reached a mean clinical score of 3 or greater at termination.

Clinical disease score

Normal paw = 0

Wrist/ankle swollen = 1

Wrist/ankle and pad swollen = 2

Wrist/ankle, pad and digits swollen = 3

2.4.2 CII-induced hypersensitivity

Sensitisation to CII induces an immune response involving both T and B cells and on re-exposure to the same antigen hypersensitivity reactions occur. Hypersensitivity reactions can be easily quantified by measuring swelling at the site of re-exposure. CII hypersensitivity was assessed using a similar protocol reported by Farmer *et al.* (1986). Fourteen days post-sensitisation mice were anaesthetised and challenged with

CII to assess hypersensitivity reactions. One day prior to challenge chick CII was dissolved in PBS at a concentration of 0.8mg/mL and constantly rolled overnight in the dark at 4°C. Mice were injected with 25µL of the solution containing 20µg of CII (unless otherwise stated) into the right ear or PBS only into the left ear as a negative control. Ear thickness was measured in mm prior to challenge (0 hour) and at 6 and 24 hours post-challenge with callipers to assess swelling. Experiments were terminated 24 hours post-challenge and blood was taken via cardiac puncture under terminal anaesthesia for serum.

2.4.3 Anti-CII IgG1 and IgG2a ELISA

Sensitisation to CII induces an immune response. Antibodies against CII such as anti-CII IgG1 and anti-CII IgG2a form part of this response and can be easily detected using sandwich ELISA techniques as detailed below. The presence of anti-CII IgG1 and anti-CII IgG2a were determined in serum samples derived from mice 15 days post-CII sensitisation (unless otherwise stated).

1. CII was dissolved in PBS to give a 1mg/mL solution and constantly rolled overnight in the dark at 4°C. This was then stored at -70°C as a stock solution. Stock was defrosted when needed and diluted to a working concentration of 5µg/mL in PBS. ELISA plates (96 well) were then coated with 100µL per well of CII solution. Plates were then covered and stored overnight at 4°C.
2. The next day plates were aspirated and washed twice with PBS on an automated plate washer.
3. Plates were blocked with 1% BSA in PBS (200µL per well) and placed on a

plate shaker for 30 minutes at room temperature.

4. Plates were aspirated and washed twice with PBS on an automated plate washer.

5. Serum samples were diluted 1 in 10,000 in PBS and 100µL aliquots of each sample were added to plates in duplicate.

6. Plates were placed on a plate shaker for 1 hour at room temperature and then washed twice with PBS.

7. IgG1 and/or IgG2a – HRP conjugated antibodies were diluted from stock 1 in 5,000 in PBS containing 1% BSA and 100µL per well was added to the plates.

8. Plates were placed on a plate shaker for 1 hour at room temperature and then washed four times with PBS.

9. TMB substrate, 100µL per well, was added to the plates.

10. The reaction was stopped after 10 minutes with 100µL 1N sulphuric acid and read on a plate reader at 450 and 630 nM wavelength.

2.4.4 Serum Amyloid A (SAA) assay

Serum samples prepared from the blood of sensitised mice, taken at various time points, were analysed using an SAA detection kit (Biognosis, Tridelta Development Ltd, Maynooth, Ireland) following the manufacturers protocol as detailed below. The amount of SAA in the serum is determined by sandwich ELISA. A monoclonal antibody specific for SAA was coated directly onto the surface of 96-well microtitre plates and samples were added to the wells with a HRP labelled anti-SAA monoclonal antibody. SAA present in the samples is captured between the two monoclonal antibodies. TMB was added to the wells to produce a colorimetric

reaction which is easily quantified and is directly proportional to the amount of SAA present.

Reagent preparation

Diluent buffer – 1 part of diluent buffer concentrate was diluted in 9 parts distilled water.

Wash buffer – 1 part of wash buffer concentrate was diluted in 19 parts distilled water.

SAA standard – SAA calibrator standard was dissolved in 200µL of distilled water with vigorous mixing to ensure the standard was completely dissolved. Serial dilutions were prepared with 50µL of SAA standard diluted in 200µL of diluent buffer and mixed well, then serially diluted four times by taking 125µL of previous solution and adding to 125µL diluent buffer. This gave concentrations of 0.950, 0.475, 0.237, 0.118, 0.059 and 0.000µg/mL (diluent buffer only) for the standard curve.

1. 50µL of anti-SAA - HRP conjugate was added to each well.
2. Serum samples were diluted to the desired concentration in diluent buffer and ranged from a 1 in 200 to a 1 in 15,000 dilution.
3. 50µL of diluted standards and serum samples were added in duplicate to each well. The sides of the plates were gently tapped to mix.
4. The plates were covered with a plate sealer and incubated for 1 hour at 37°C.
5. The plates were aspirated and washed four times with wash buffer.
6. 100µL of TMB substrate was added to each well.

7. Plates were incubated at room temperature for 15 minutes.

8. 100μL of stop solution was added to each well and mixed by gently tapping the sides of the plates.

9. Absorbance of the plates was read at 450 and 630nm wavelength on a plate reader.

SAA levels were calculated as μg/mL using the standard curve. A typical standard curve is shown in Figure 2.1.

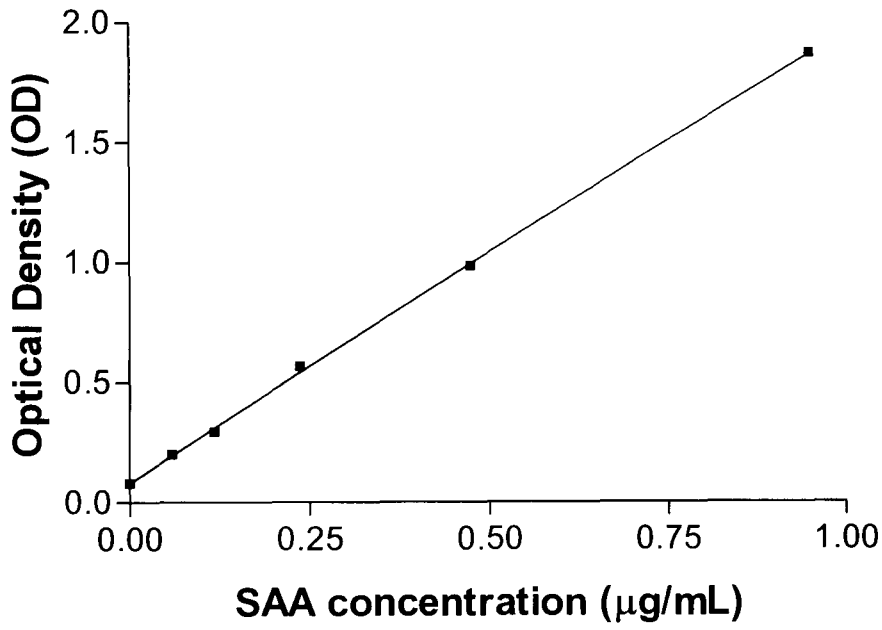


Figure 2.1 Serum amyloid A standard curve

2.4.5 CII stimulated CD4$^+$ T cell proliferation

Cell harvest

$CD4^+$ T cells isolated from CII sensitised mice can be used to assess the cellular immune response. Re-stimulation of these cells *ex vivo* with CII induces proliferation. The $CD4^+$ T cell proliferation assay in this thesis was adapted from a single cell suspension assay described by Mauri *et al.* (1996). Inguinal lymph nodes were dissected out from normal male DBA/1 mice or from mice 14 days after sensitisation with either complete Freund's adjuvant (CFA) or collagen II (CII) in CFA and placed in cell culture medium. Lymph nodes were pooled from each experimental group. In a class II fume hood lymph nodes were pushed through a 70 micrometer (μm) mesh with a 2mL syringe plunger end and cells were collected in a petri dish. The mesh was washed out with medium to ensure as many cells as possible had passed through. Using a plastic pipette, cell suspensions were transferred from the petri dish to a 15mL falcon tube and centrifuged for 5 minutes at 410xg. Supernatants were poured into verkon and disposed of down the sink at the end of experiments. The tubes were agitated to break up the cell pellets and cells were resuspended in 1mL of culture medium. The cells were stained by diluting 1 in 100 in trypan blue in a 96 well plate. Cells were stained for 2-3 minutes then counted. Dead cells take up blue dye and cells that appeared white were counted. Counts were conducted using a graticual and microscope, which gave counts at $1x10^4$ and were adjusted for the dilution factor giving counts at $1x10^6$.

$CD4^+$ cell separation

$CD4^+$ MACS separation kit (Miltenyi Biotec, Bisley, U.K.) was used following the manufacturers protocol as detailed below. This is a negative selection kit where $CD4^+$ T cells are isolated by removal of non-$CD4^+$ T cells. Non-$CD4^+$ T cells are indirectly magnetically labelled with a cocktail of biotin conjugated mAbs, as primary labelling reagent, and anti-biotin mAbs conjugated to microbeads, as secondary labelling reagent. Magnetically labelled non-$CD4^+$ T cells are removed by retaining them on a MACS column in the magnetic field of a MACS separator, while the $CD4^+$ T cells pass through the column.

When using this magnetic labelling technique it was essential to work fast and keep cells cold, using chilled solutions only.

Magnetic labelling

1. Harvested cells were centrifuged at 410xg for 10 minutes.
2. Supernatants were removed and cell pellets resuspended in 40µL of MACS buffer per $1x10^7$ cells.
3. 10µL of biotin-antibody cocktail was added per $1x10^7$ cells.
4. Cells were mixed well and incubated for 10 minutes at 4-8°C.
5. 30µL of MACS buffer and 20µL of anti-biotin microbeads were added per $1x10^7$ cells.
6. Cells were mixed well and incubated for 15 minutes at 4-8°C.
7. Cells were washed with MACS buffer by adding 10-20 times the labelling volume and centrifuged at 410xg for 10 minutes.
8. Supernatant was completely removed and placed into verkon.

9. The cell pellets were resuspended in 500μL of buffer per $1x10^8$ cells (for less cells 500μL was used).

Magnetic separation

LS+ MACS columns were used for positive cell separation.

1. LS+ columns were placed in the MACS separator with 15mL falcon tubes underneath.
2. MACS columns were prepared by rinsing with 3mL of MACS buffer, which was collected in the 15mL falcon tubes. Buffer was then disposed of and new falcon tubes were placed under the MACS columns to collect cells.
3. Cell suspensions were applied to MACS columns and the fraction of unlabelled $CD4^+$ cells were collected in the 15mL falcon tubes.
4. MACS columns were washed four times with 3mL of MACS buffer, which was collected in the same tubes.
5. Cells were centrifuged and counted by staining as described above.
6. $CD4^+$ cells were resuspended at 5 x 10^6 cells/mL in cell culture medium.

APC cell preparation

1. Spleens were removed from naïve mice and pooled in cell culture medium.
2. Spleens were mashed with a syringe plunger and passed through a 70μm mesh into petri dishes.
3. Cells were centrifuged at 410xg for 5 minutes and supernatants were discarded into verkon.
4. 3mL of sterile RBC lysis buffer was added to the cell pellets for 3 minutes.

5. The volume was made up to 10mL with cell culture medium and centrifuged at 410xg for 5 minutes.

6. Cells were resuspended in 1mL of cell culture medium and counted by diluting 1 in 100 in trypan blue stain (counts at $1x10^6$).

7. Cells were resuspended at $5x10^7$ cells/mL in cell culture medium.

8. A 0.5mg/mL solution of mitomycin C was made up by adding 4mL of sterile PBS to one vial of mitomycin C, which was agitated and filtered through a 20μm syringe filter.

9. 100μL mitomycin C solution was added per 1mL of cells.

10. Cells were incubated at 37°C for 35 minutes.

11. Cells were centrifuged and washed four times in cell culture medium.

12. Cells were resuspended at $2x10^7$ cells/mL in cell culture medium.

Unless otherwise stated 50μL of $CD4^+$ cells ($2.5x10^5$ per well) were plated out with 50μL of APCs ($1x10^6$ per well) into 96 well plates. In some experiments drugs were diluted in sterile PBS and assessed *in vitro* at a concentration of 0.1, 1, 10 and 100μg/mL for mAbs or 0.1, 1 and 10μM for cyclosporin A.

Prior to cell harvest, chick CII was dissolved overnight at 4°C in PBS at 0.5mg/mL. CII solution was then denatured in a water bath at 60°C for 2-3 hours and allowed to cool. This was done in advance and the CII solution was frozen in aliquots. Aliquots were defrosted as required on the day of cell culture. To stimulate cell proliferation 20μL of the CII solution was added to some wells at a concentration of 50μg/mL (unless otherwise stated). The volume of each well was then topped up to 200μL with culture medium. Cells were placed in a humidified 37°C CO_2 incubator for 72 hours (unless otherwise stated).

To assess cell proliferation 20μL ^{3}H thymidine (1μCi) was added to each well for the last 6 hours of culture. Plates were frozen and then defrosted at a later date for analysis of cell proliferation. Cells were harvested from defrosted plates by an automated cell harvester onto filter paper and dried in the oven for 30 minutes. Radionuclide uptake was measured by liquid scintillation counting on a β counter and total counts per minute (cpm) were obtained. In some experiments ^{3}H thymidine was not added and cell culture supernatants (100μL) were removed for cytokine analysis as described below.

Radioactive material was disposed of in designated bins and sinks and recorded on Isostock (an in-house tracking system).

2.4.6 IL-4, IL-17 and IFNγ ELISA

$CD4^{+}$ T cell cultures were set up as described above. Cell culture supernatants taken at various time points were analysed for levels of IL-4, IL-17 and IFNγ using DuoSet® ELISA development system kits (R&D Systems, Abingdon, U.K.) following the manufacturer's protocol as detailed below. These kits use sandwich ELISA techniques with a colorimetric endpoint to measure the amount of cytokine present in the supernatant.

Reagent preparation

All reagents were brought to room temperature before use.

Wash buffer – 0.05% Tween 20 in PBS, pH 7.2.

Reagent diluent – 1% BSA in PBS.

Capture antibodies for IL-4, IL-17 and IFNγ were reconstituted with 1mL of PBS.

Detection antibodies for IL-4, IL-17 and IFNγ were reconstituted with 1mL of reagent diluent.

Recombinant mouse IL-4, IL-17 and IFNγ standards were reconstituted with 0.5mL reagent diluent and allowed to sit for 15 minutes with gentle agitation. The manufacture's serial dilutions were followed to give a seven-point standard curve, this varied for each kit.

Streptavidin-HRP was diluted 200 fold to give the correct working concentration.

Substrate solution was mixed in a 1:1 ratio of colour reagent A and B.

Sample preparation

Supernatants from cell cultures were used neat for IL-4 and IFNγ assays and diluted 1 in 10 in reagent diluent for IL-17 assays.

1. The required number of 96 well ELISA plates were coated with 100µL per well of the appropriate capture antibody diluted to the working concentration. Plates were then sealed with an adhesive strip and incubated overnight at room temperature.
2. The next day, plates were aspirated and washed three times with wash buffer on an automated plate washer.
3. Plates were blocked by adding 300µL of reagent diluent to each well and incubated at room temperature for a minimum of 1 hour.
4. Plates were washed as in step 2.
5. 100µL of sample or appropriate standard was added to plates and incubated for 2 hours at room temperature.
6. Plates were washed as in step 2.

7. 100μL of the appropriate detection antibody, diluted to the working concentration, was added to each well and covered with a new adhesive strip and incubated for 2 hours at room temperature.

8. Plates were washed as in step 2.

9. 100μL of streptavidin-HRP, diluted to the working concentration, was added to each well and incubated for 20 minutes at room temperature.

10. Plates were washed as in step 2.

11. 100μL of prepared substrate solution was added to each well and incubated for 20 minutes at room temperature.

12. 50μL of stop solution was added to each well and plates were gently tapped to ensure thorough mixing.

13. Absorbance of the plates was read at 450 and 630nm wavelength on a plate reader.

The levels of IL-4, IL-17 and IFNγ were calculated as μg/mL using standard curves. A typical standard curve is shown in Figure 2.2.

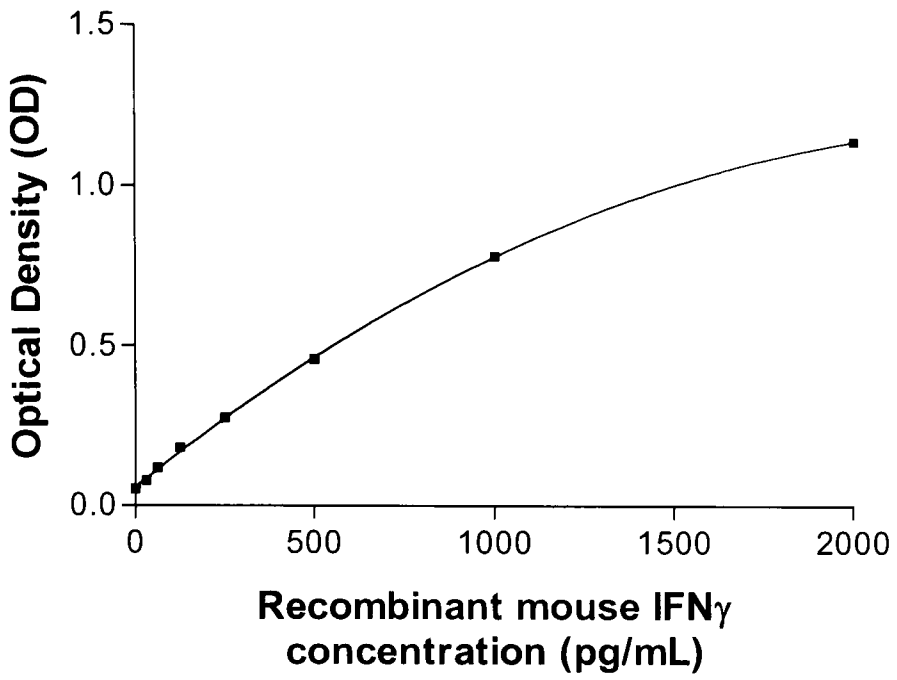


Figure 2.2 Interferon gamma (IFNγ) standard curve

2.4.7 IL-4, IL-17 and IFNγ ELISpot

ELISpot assays have been designed to detect cytokine secreting cells at the single cell level. These assays use the sandwich ELISA technique. $CD4^+$ T cells and APCs were isolated as described above and incubated in 96-well plates pre-coated with a monoclonal antibody specific for either IL-4 or IL-17 or IFNγ. During the incubation period cytokines released from cells are bound by the cytokine specific monoclonal antibodies. A cytokine specific biotinylated polyclonal antibody was then added to the wells. Alkaline-phosphatase conjugated to streptavidin was then added followed by a substrate solution and a blue-black coloured spot forms at the sites of cytokine localisation. Each spot represents an individual cytokine secreting cell which allows the frequency of IL-4, IL-17 and IFNγ secreting cells in culture to be determined.

Reagent preparation

Wash buffer concentrate was diluted 1 in 10 with deionised water.

Mouse IL-4, IL-17 and IFNγ positive controls were reconstituted with 0.25mL of culture medium.

Detection antibodies - 100μL of the detection antibody concentrate was added to dilution buffer 1 and mixed well.

Streptavidin-AP - 100μL of streptavidin-Ap concentrate A was added to dilution buffer 2 and mixed well.

1. 200μL of sterile culture medium was added to each well of the IL-4, IL-17 and IFNγ ELISpot microplates and incubated for 20 minutes.

2. Culture medium was aspirated from the wells and 2.5 x 10^5 $CD4^+$ T cells together with 1 x 10^5 APCs were added. Anti-IL-17 and anti-IFNγ mAbs were also added to some wells at a concentration of 1, 10 and 100μg/mL. In some experiments recombinant mouse IL-17, IFNγ and IL-4 were added to some wells at a concentration of 1, 10 and 100ng/mL. Some wells received 50μg/mL of denatured CII.

3. Cells were cultured in a humidified 37°C CO_2 incubator for 24 or 72 hours (as stated).

4. Each well was aspirated and plates were washed four times with wash buffer on an automated plate washer.

5. 100μL of detection antibody was added to each well and incubated overnight at 2-8°C.

6. Plates were washed as in step 4.

7. 100μL of diluted streptavidin-AP was added to each well and incubated for 2 hours at room temperature.

8. Plates were washed as in step 4.

9. 100μL of BCIP/NBT chromogen was added to each well and incubated for 1 hour at room temperature protected from light.

10. The chromogen solution was discarded and plates were rinsed with deionised water. Plates were inverted and tapped to remove excess water. The flexible plastic underdrain was removed from the bottom of the plates. Plates were completely dried by leaving them at room temperature for 1 hour.

11. Plates were analysed for the number of IL-4, IL-17 or IFNγ positive spots using an automated ELISpot plate reader.

The number of IL-4, IL-17 and IFNγ positive spots per total cells plated out were calculated.

2.4.8 Flow cytometric analysis

Depletion of $CD4^+$ and $CD8^+$ T cells after *in vivo* anti-$CD4^+$ and anti-$CD8^+$ treatment was confirmed using flow cytometric analysis. These antibodies have previously been shown to successfully deplete $CD4^+$ and $CD8^+$ T cells respectively (Cobbold *et al.*, 1984). At termination of some studies inguinal lymph nodes were dissected out and cell suspensions were prepared as described for cell harvest above. Cells were washed in 3mL of ice-cold FACS buffer and centrifuged at 410xg for 5 minutes. Cells were then resuspended in 1mL of FACS buffer and 100μL of each sample was removed and placed in 5mL polypropylene Falcon tubes. Anti-CD4 FITC conjugated and anti-CD8 PE conjugated antibodies were added to cell suspensions at a concentration of 10μg/mL and 4μg/mL respectively. Samples were incubated for 30 minutes on ice. The cells were then washed twice in 3mL of FACS buffer and resuspended in 0.5mL

of FACS buffer. Analysis of the cells was carried out on a FACScalibur flow cytometer equipped with CellQuest software. A decrease in the detection of fluorescently labelled anti-CD4 and anti-CD8 in cells from treated mice, as compared to cells from vehicle treated CII/CFA control mice, indicated depletion of $CD4^+$ and $CD8^+$ T cells respectively.

2.5 Data handling

Data handling and statistical analysis was conducted on GraphPad Prism V3.03.

Chapter three

Drug effects in the collagen-induced arthritis (CIA) model

3.1 Introduction

CIA is a long-term pre-clinical model of RA and is widely used to assess anti-arthritic drug effects. Many of the drug targets under investigation in this thesis have been demonstrated to play a role in models of arthritis. However, protocols used to induce CIA vary from one research establishment to another and many of the in-house mAbs have not previously been shown to be active in the CIA model. It was therefore essential to evaluate these drugs in the CIA model in-house to determine their anti-arthritic activity.

The majority of the mAbs used in this thesis have pM affinities for their targets as outlined in Chapter 2, Table 2.1. In addition, these mAbs were assessed in *in vitro* assays and pharmacokinetic studies prior to the initiation of CIA experiments. Results from these studies identified the concentrations of mAb needed for biological activity and the doses and frequencies required to achieve these levels in the blood. Leflunomide is a known anti-arthritic agent, however, its effect in the murine CIA model is not well reported, therefore a dose was chosen based on activity in other animal models of RA (Thoss *et al.*, 1996). To try to avoid any adverse effects of leflunomide, a relatively low dose which was anticipated to be effective was chosen. Dose and frequency of administration for each drug are stated in the results section.

Using these drugs as pharmacological tools, the underlying mechanisms involved in CIA were investigated. Validation of the CIA model was conducted in this part of the work with the clinically relevant anti-arthritic drug leflunomide. In addition, mAbs targeting TNFα, IL-1β and IL-6, cytokines proven to be important in RA, were utilised for further validation and to determine the cytokine dependencies within the model. Anti-$CD4^+$, anti-$CD8^+$, anti-CD40L and anti-LFA-1 mAbs were used to give an insight into the immunological processes underlying the disease.

To determine the role of the novel target IL-17 in the CIA model an anti-IL-17 mAb was used. A comparison between the anti-arthritic effects of anti-IL-17 treatment and the other drugs can then be made. Such a comparison should identify the potential of targeting IL-17 as a therapy.

3.2 Aim

The aim of this chapter was to assess the anti-arthritic properties of a range of drugs in the CIA model. Drug effects in the subsequent short-term mechanistic models can then be compared with their anti-arthritic activity in this chapter and the predictive nature of these models can be determined.

3.3 Results

In these experiments data is presented as mean clinical score per group on a daily basis and mean area under the curve (AUC) of the clinical score. AUC was calculated using the statistics software on GraphPad Prism V3.03. These readouts show the progression of disease over time and the overall disease severity respectively. The anti-arthritic effect of the drugs was assessed by their ability to reduce these readouts relative to vehicle treated controls. Statistical analysis of data was conducted using the Mann Whitney test and $p<0.05$ was considered significant.

3.3.1 $CD4^+$ and $CD8^+$ T cell depletion

The role of $CD4^+$ and $CD8^+$ T cells in the CIA model was assessed using mAbs capable of depleting these cells (Figure 3.1). Anti-$CD4^+$ and anti-$CD8^+$ mAb treatment was assessed against the same control group. In the vehicle control group, mice showed signs of arthritis 26 days after sensitisation. Arthritis in this group got progressively worse over time resulting in a mean clinical score of 6.14 ± 1.10 on Day 54 when the study was terminated. CIA mice treated with anti-$CD4^+$ mAb showed a complete abrogation of arthritis (Figure 3.1a) with a statistically significant 100% inhibition in AUC of the clinical score (Figure 3.1b). Mice treated with anti-$CD8^+$ mAb showed signs of arthritis 17 days post-sensitisation with the mean clinical score increasing to 4.93 ± 0.84 on Day 54 (Figure 3.1a). Anti-$CD8^+$ treatment caused a 19% reduction in AUC of the clinical score but this was not statistically significant (Figure 3.1b).

Depletion of $CD4^+$ and $CD8^+$ T cells was confirmed in the lymph nodes of treated mice at termination of the experiment by FACS analysis (Figure 3.2). These mAbs have previously been shown to deplete their target cells in-house (historical data) and

in the literature in mice (Cobbold *et al.*, 1984). Thus staining of CD4 and CD8 only was considered satisfactory to assess depletion. Anti-$CD4^+$ treatment caused a statistically significant 72% ($p<0.01$) depletion of $CD4^+$ T cells in the lymph nodes as compared to vehicle treated mice. Anti-$CD8^+$ treatment resulted in a statistically significant 80% ($p<0.05$) depletion of $CD8^+$ T cells in the lymph nodes as compared to vehicle treated mice. Depletion of $CD4^+$ and $CD8^+$ T cells was also seen in the blood (data not shown).

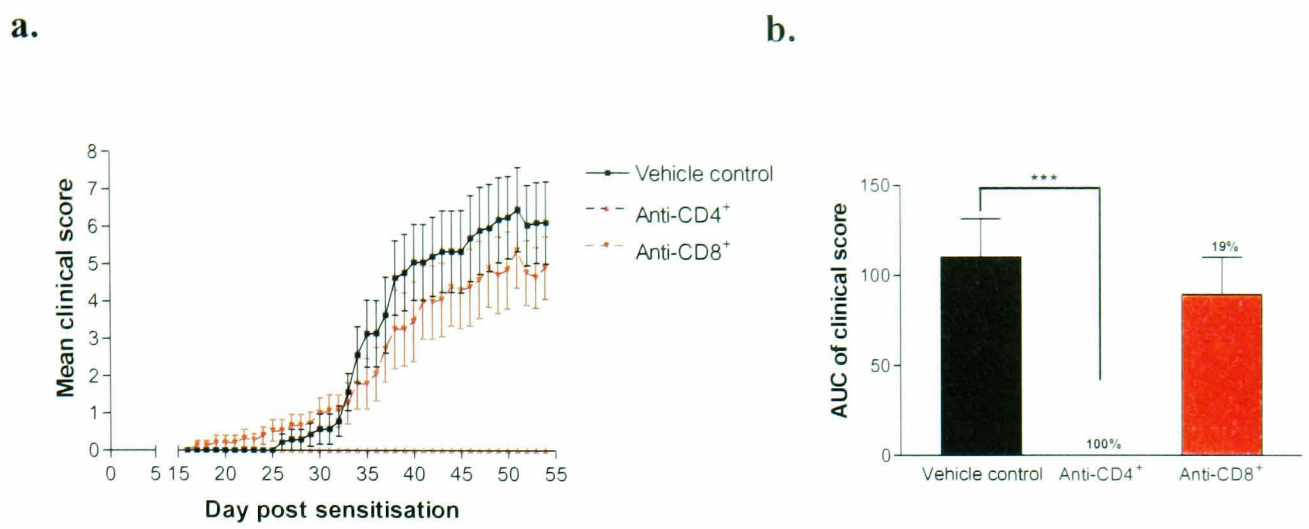
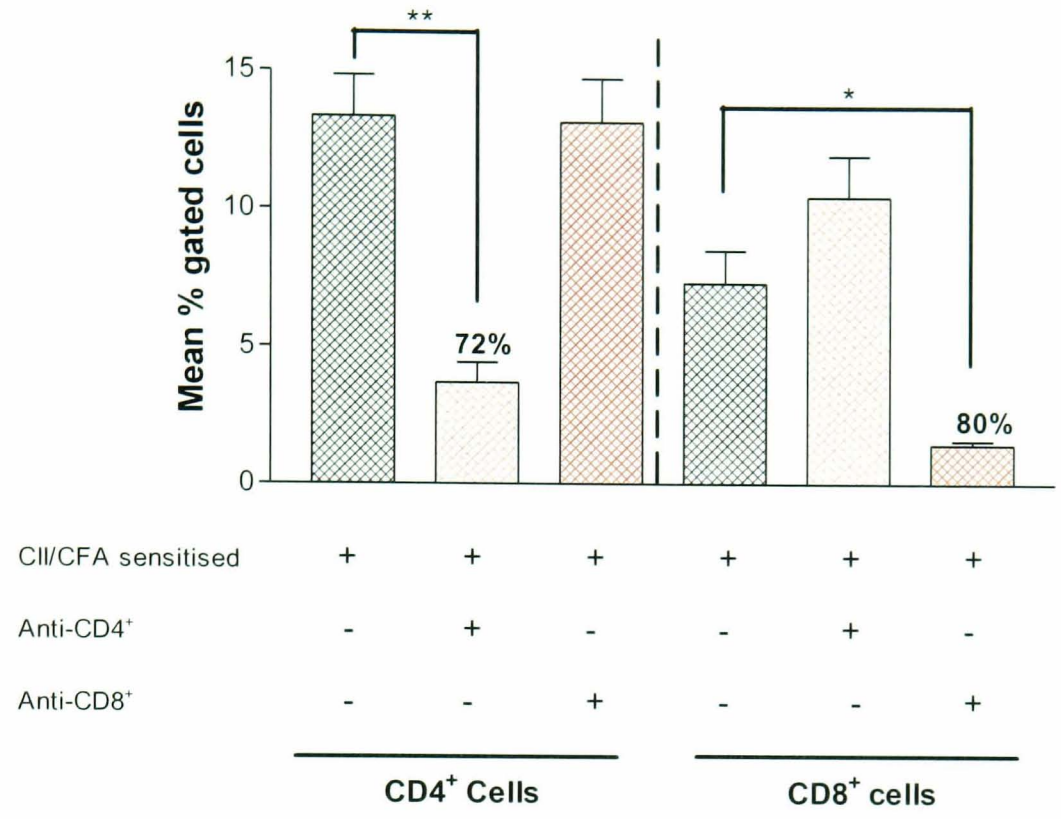


Figure 3.1 Effect of prophylactic anti-CD4$^+$ and anti-CD8$^+$ antibody treatment on collagen-induced arthritis.

Male DBA/1 mice were sensitised by intradermal injection of 100µg collagen II (CII) in 0.1M acetic acid and complete Freund's adjuvant at the base of the tail, 14 days post-sensitisation animals were boosted with 100µg CII in incomplete Freund's adjuvant. Paws were scored daily for clinical signs of arthritis on a 0-3 scale taking into account swelling of the wrist/ankle, pad and digits. Data presented as mean clinical score (a) and AUC of the clinical score (b). Some animals were dosed with anti-mouse CD4$^+$ or anti-mouse CD8$^+$ antibodies 10mg/kg s.c. on three consecutive days prior to sensitisation and thereafter once a week. Data presented as mean ± s.e.m, n = 15 per group, *** $p<0.001$ statistically significant from indicated control. The numbers indicate percent inhibition of CIA with anti-CD4$^+$ and anti-CD8$^+$ antibody

a.



b. Control **c. Anti-CD4$^+$** **d. Anti-CD8$^+$**

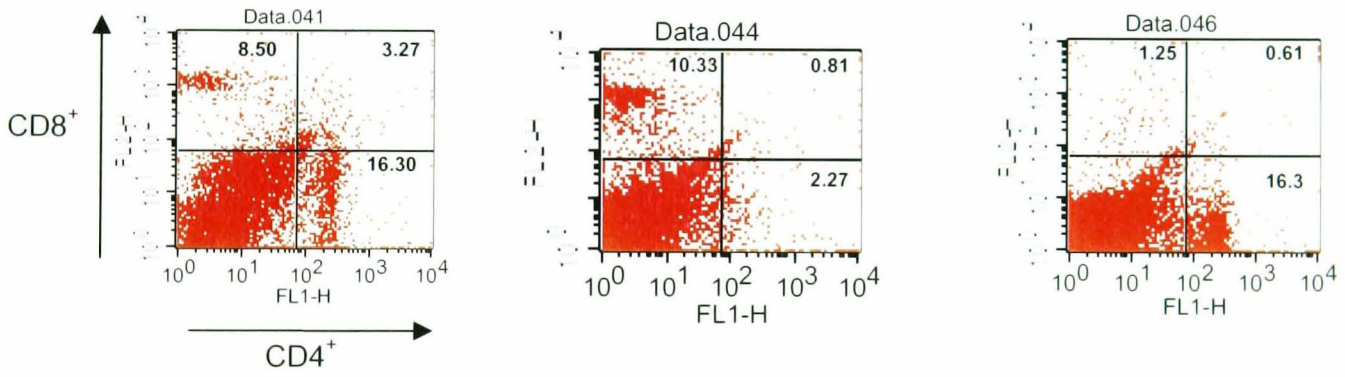


Figure 3.2 Confirmation of CD4$^+$ and CD8$^+$ T cell depletion in lymph nodes.

Male DBA/1 mice were sensitised by intradermal injection of 100µg collagen II (CII) in 0.1M acetic acid and complete Freund's adjuvant at the base of the tail, 14 days post-sensitisation animals were boosted with 100µg CII in incomplete Freund's adjuvant. Some animals were dosed with anti-mouse CD4$^+$ or anti-mouse CD8$^+$ antibodies 10mg/kg s.c. on three consecutive days prior to sensitisation and thereafter once a week. At termination of the experiment (Day 54) mice were killed and inguinal lymph nodes were removed. Single cell suspensions were prepared and cells

were stained with anti-CD4 FITC conjugated and anti-CD8 PE conjugated antibodies for flow cytometric analysis as described in Chapter 2. The mean percentage of gated cells was calculated for each group of mice (a). A representative fluorescence activated cell sorter plot is shown for vehicle treated CFA/CII sensitised controls (b), CFA/CII sensitised anti-$CD4^+$ treated (c) and CFA/CII sensitised anti-$CD8^+$ treated mice (d). Data presented as mean ± s.e.m, n = 15 per group, ** $p<0.01$ and * $p<0.05$ statistically significant from indicated control. The numbers indicate percent depletion of $CD4^+$ and $CD8^+$ T cells as compared to indicated control (a) and the percent gated cells (b, c and d).

3.3.2 Leflunomide

In order to demonstrate that a clinically relevant drug, used in the treatment of RA, is active in the CIA model, leflunomide was assessed (Figure 3.3). CIA mice that were administered vehicle started to show signs of arthritis on Day 16 post-sensitisation. Disease severity in this group increased to give a mean clinical score of 5.27 ± 0.95 at the end of the experiment on Day 36. Mice treated with leflunomide also showed signs of arthritis on Day 16, however disease progression was inhibited when compared to vehicle control animals, with a mean clinical score of 1.47 ± 0.52 on Day 36 (Figure 3.3a). This reduction in disease severity with leflunomide gave a statistically significant 68% inhibition in AUC of the clinical score (Figure 3.3b).

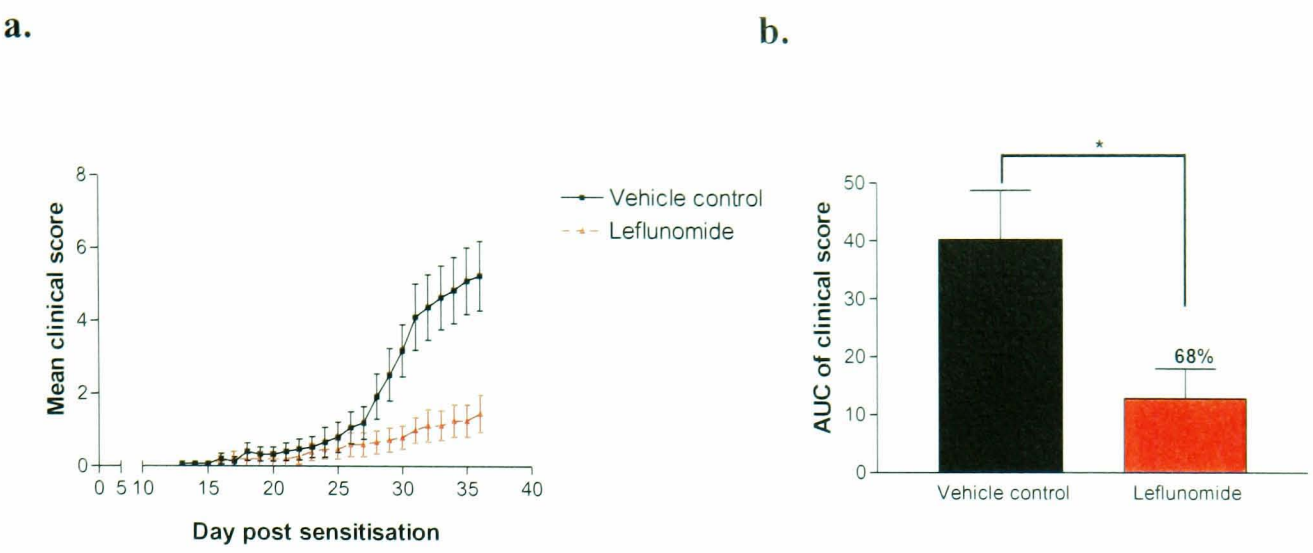


Figure 3.3 Effect of prophylactic leflunomide treatment on collagen-induced arthritis.

Male DBA/1 mice were sensitised by intradermal injection of 100μg collagen II (CII) in 0.1M acetic acid and complete Freund's adjuvant at the base of the tail, 14 days post-sensitisation animals were boosted with 100μg CII in incomplete Freund's adjuvant. Paws were scored daily for clinical signs of arthritis on a 0-3 scale taking into account swelling of the wrist/ankle, pad and digits. Data presented as mean clinical score (a) and AUC of the clinical score (b). Some animals were dosed orally with leflunomide 3mg/kg daily from one day prior to sensitisation. Data presented as mean ± s.e.m, n = 15 per group, * $p<0.05$ statistically significant from indicated control. The number indicates percent inhibition of CIA with leflunomide treatment.

3.3.3 Anti-CD40L

The binding of CD40L on T cells to CD40 on B cells is essential in adaptive immunity. The effect of blocking this interaction in the CIA model was assessed using a mAb against CD40L (Figure 3.4). Vehicle control mice showed signs of arthritis on Day 21 with the severity of disease increasing over time giving a mean clinical score of 3.20 ± 0.87 on Day 48. In mice treated with anti-CD40L attenuation of disease was seen, with signs of arthritis being delayed until Day 30 and the mean clinical score only increasing to 0.57 ± 0.40 on Day 48 (Figure 3.4a). Treatment with anti-CD40L resulted in a statistically significant 81% inhibition in AUC of the clinical score (Figure 3.4b).

3.3.4 Anti-LFA-1

Another fundamentally important cellular interaction in the initiation of an immune response is that of the T cell and the APC. This interaction occurs partly through LFA-1, expressed on T cells, binding its ligand ICAM, expressed on APCs. In order to block this interaction and identify its role in the CIA model, a mAb against LFA-1 was assessed (Figure 3.5). CIA mice that received vehicle started to show signs of arthritis on Day 27 which got progressively worse over the course of the experiment resulting in a mean clinical score of 3.67 ± 0.84 at termination on Day 56. Mice treated with anti-LFA-1 showed signs of arthritis on Day 30 and followed a similar disease pattern to vehicle control animals, with a mean clinical score of 2.73 ± 0.88 at the end of the study (Figure 3.5a). The AUC of the clinical score was analysed for each group and anti-LFA-1 showed a 38% inhibition in disease, but this was not statistically significant (Figure 3.5b).

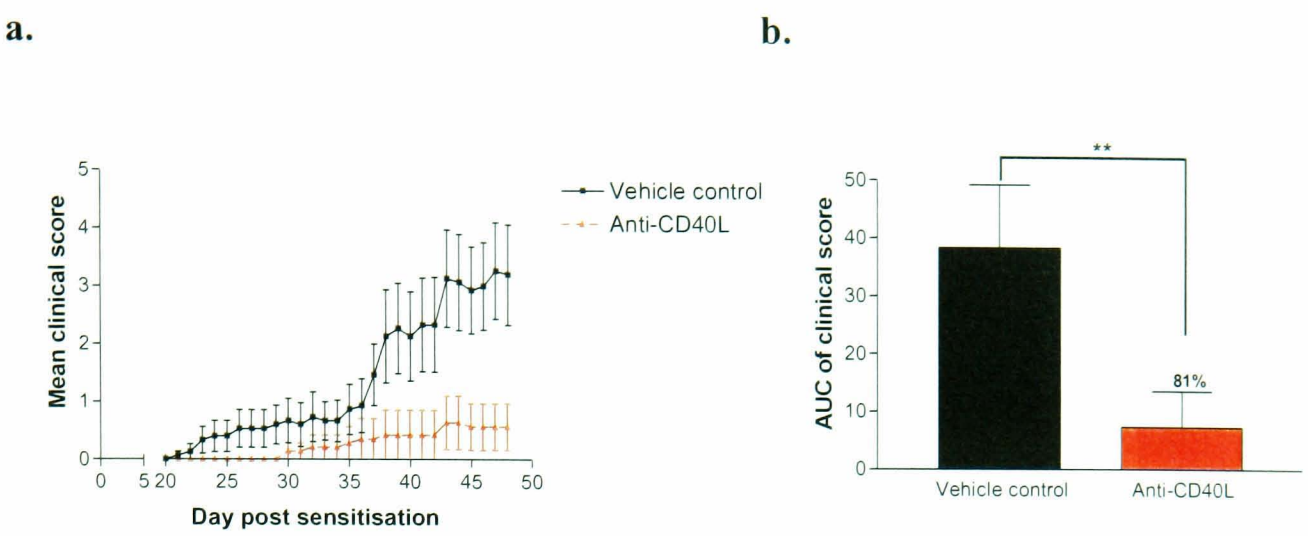


Figure 3.4 Effect of prophylactic dosing with anti-CD40L Fab PEG antibody on collagen-induced arthritis.

Male DBA/1 mice were sensitised by intradermal injection of 100µg collagen II (CII) in 0.1M acetic acid and complete Freund's adjuvant at the base of the tail, 14 days post-sensitisation animals were boosted with 100µg CII in incomplete Freund's adjuvant. Paws were scored daily for clinical signs of arthritis on a 0-3 scale taking into account swelling of the wrist/ankle, pad and digits. Data presented as mean clinical score (a) and AUC of the clinical score (b). Some animals were dosed with anti-mouse CD40L Fab PEG antibody 100mg/kg s.c. once a week from one day prior to sensitisation. Data presented as mean ± s.e.m, n = 15 per group, ** $p<0.01$ statistically significant from indicated control. The number indicates percent inhibition of CIA with anti-CD40L Fab PEG antibody treatment.

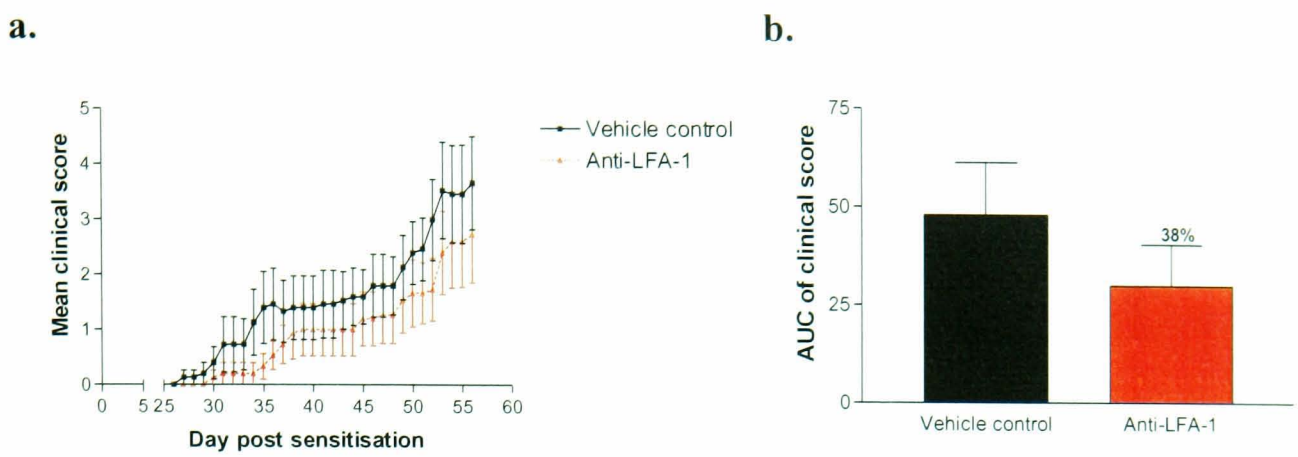


Figure 3.5 Effect of prophylactic dosing with anti-LFA-1 antibody on collagen-induced arthritis.

Male DBA/1 mice were sensitised by intradermal injection of 100μg collagen II (CII) in 0.1M acetic acid and complete Freund's adjuvant at the base of the tail, 14 days post-sensitisation animals were boosted with 100μg CII in incomplete Freund's adjuvant. Paws were scored daily for clinical signs of arthritis on a 0-3 scale taking into account swelling of the wrist/ankle, pad and digits. Data presented as mean clinical score (a) and AUC of the clinical score (b). Some animals were dosed with anti-mouse LFA-1 antibody 30mg/kg s.c. once a week from one day prior to sensitisation. Data presented as mean ± s.e.m, n = 15 per group. The number indicates percent inhibition of CIA with anti-LFA-1 antibody treatment.

3.3.5 Anti-TNFα

In order to assess the role of TNF in the CIA model a mAb against TNFα was assessed (Figure 3.6). Vehicle control mice showed signs of arthritis on Day 19. with the mean clinical score rising over the course of the experiment to 4.20 ± 0.79 on Day 45. Anti-TNFα treatment delayed disease onset until Day 30 and attenuated signs of arthritis throughout the study, resulting in a mean clinical score of 1.33 ± 0.46 on Day 45 (Figure 3.6a). Anti-TNFα treatment caused a corresponding statistically significant 82% inhibition in the AUC of the clinical score (Figure 3.6b).

3.3.6 Anti-IL-1β

A mAb directed against IL-1β was utilised to assess the role that this cytokine plays in CIA (Figure 3.7). Signs of arthritis in the vehicle control group were seen on Day 19 post-sensitisation and disease severity increased throughout the study resulting in a mean clinical score of 6.07 ± 0.99 on Day 40. Anti-IL-1β treatment abrogated signs of CIA, with a delay in disease onset to Day 39 and a mean clinical score of 0.33 ± 0.27 on Day 40 (Figure 3.7a). This translated into a statistically significant 99.6% inhibition in AUC of the clinical score when mice were treated with anti-IL-1β (Figure 3.7b).

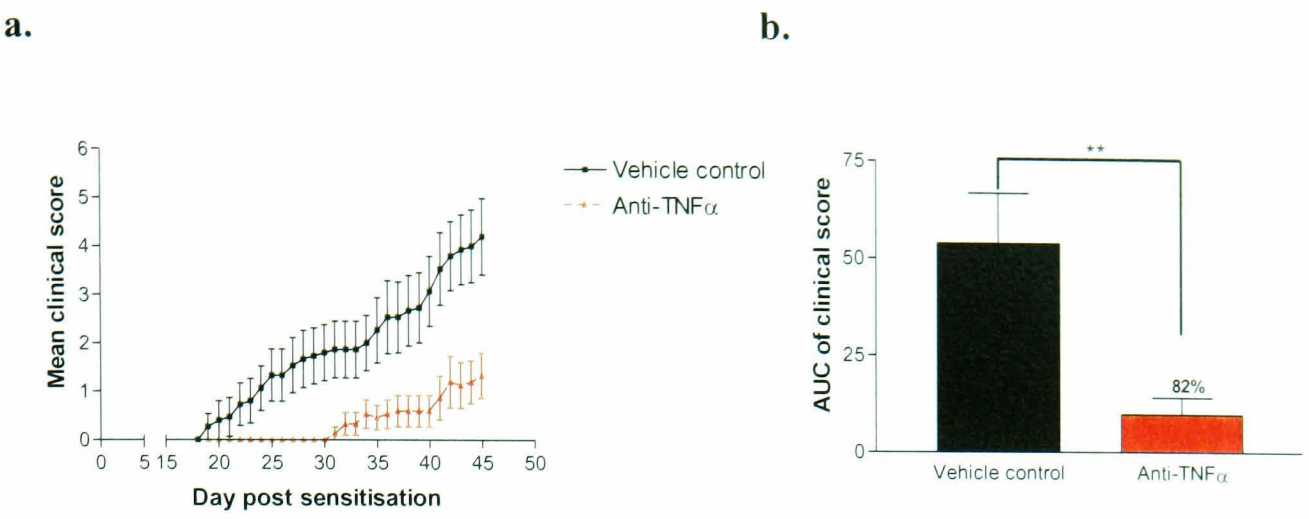


Figure 3.6 Effect of prophylactic dosing with anti-TNFα antibody on collagen-induced arthritis.

Male DBA/1 mice were sensitised by intradermal injection of 100μg collagen II (CII) in 0.1M acetic acid and complete Freund's adjuvant at the base of the tail, 14 days post-sensitisation animals were boosted with 100μg CII in incomplete Freund's adjuvant. Paws were scored daily for clinical signs of arthritis on a 0-3 scale taking into account swelling of the wrist/ankle, pad and digits. Data presented as mean clinical score (a) and AUC of the clinical score (b). Some animals were dosed with anti-mouse TNFα antibody 100mg/kg s.c. twice a week from one day prior to sensitisation. Data presented as mean ± s.e.m, n = 15 per group, ** $p<0.01$ statistically significant from indicated control. The number indicates percent inhibition of CIA with anti-TNFα antibody treatment.

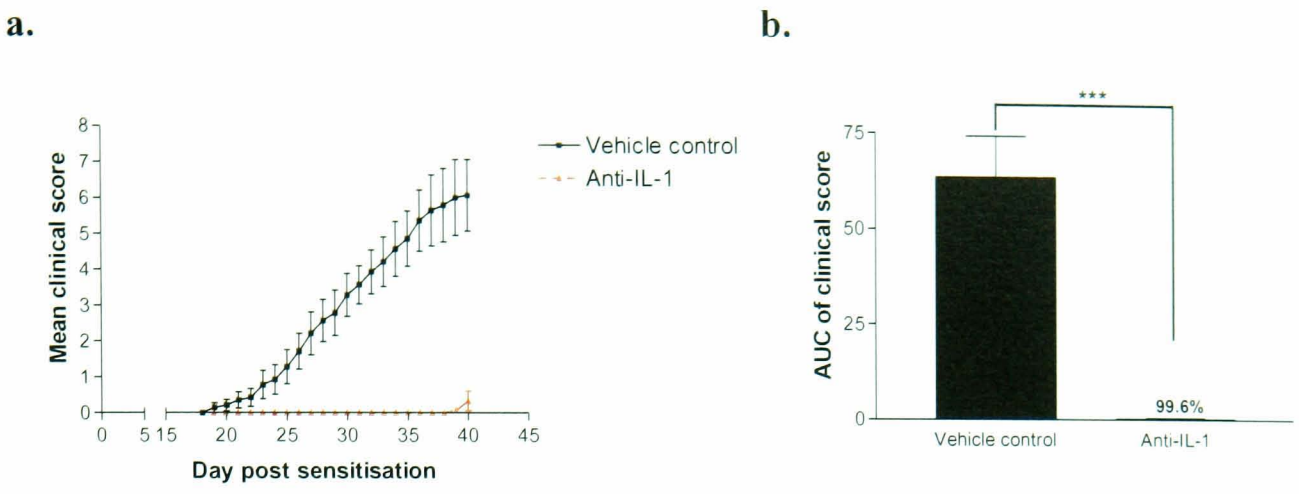


Figure 3.7 Effect of prophylactic dosing with anti-IL-1β antibody on collagen-induced arthritis.

Male DBA/1 mice were sensitised by intradermal injection of 100μg collagen II (CII) in 0.1M acetic acid and complete Freund's adjuvant at the base of the tail, 14 days post-sensitisation animals were boosted with 100μg CII in incomplete Freund's adjuvant. Paws were scored daily for clinical signs of arthritis on a 0-3 scale taking into account swelling of the wrist/ankle, pad and digits. Data presented as mean clinical score (a) and AUC of the clinical score (b). Some animals were dosed with anti-mouse IL-1β antibody 10mg/kg s.c. once a week from one day prior to sensitisation. Data presented as mean ± s.e.m, n = 15 per group, *** $p<0.001$ statistically significant from indicated control. The number indicates percent inhibition of CIA with anti-IL-1β antibody treatment.

3.3.7 Anti-IL-6

The role of IL-6 in the CIA model was investigated using a mAb directed against this cytokine (Figure 3.8). In this experiment CIA mice dosed with vehicle showed signs of arthritis on Day 19 post-sensitisation. The arthritis in this group increased with time, giving a mean clinical score of 4.67 ± 0.75 on Day 52. Anti-IL-6 treatment delayed the onset of disease until Day 42 and thereafter attenuated disease progression, as compared to vehicle controls, resulting in a mean clinical score of 1.13 ± 0.51 on Day 52 (Figure 3.8a). Anti-IL-6 treatment caused a statistically significant 87% inhibition in AUC of the clinical score in the CIA model (Figure 3.8b).

3.3.8 Anti-IL-17

To assess the effect of inhibiting a novel target in the CIA model a mAb against IL-17 was used (Figure 3.9). In this experiment vehicle control mice showed signs of arthritis on Day 20 post-sensitisation and disease severity increased over time giving a mean clinical score of 6.00 ± 0.92 on Day 53. Anti-IL-17 treatment attenuated signs of CIA, as compared to vehicle control mice, resulting in a mean clinical score of 2.27 ± 0.71 on Day 53 (Figure 3.9a). Anti-IL-17 treatment showed a statistically significant 75% inhibition in disease when the data was expressed as AUC of the clinical score (Figure 3.9b).

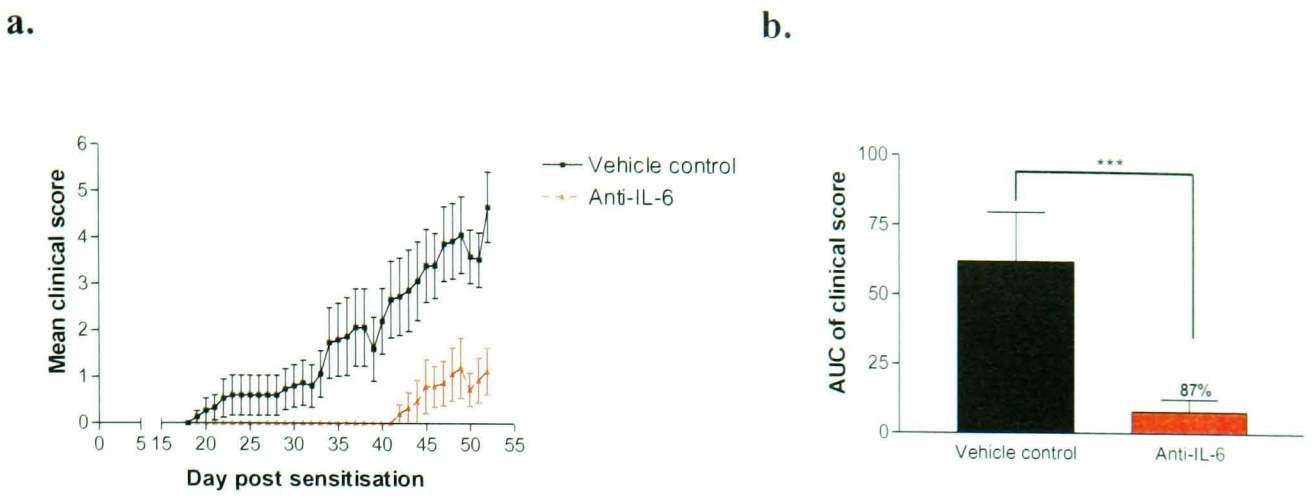


Figure 3.8 Effect of prophylactic dosing with anti-IL-6 antibody on collagen-induced arthritis.

Male DBA/1 mice were sensitised by intradermal injection of 100μg collagen II (CII) in 0.1M acetic acid and complete Freund's adjuvant at the base of the tail, 14 days post-sensitisation animals were boosted with 100μg CII in incomplete Freund's adjuvant. Paws were scored daily for clinical signs of arthritis on a 0-3 scale taking into account swelling of the wrist/ankle, pad and digits. Data presented as mean clinical score (a) and AUC of the clinical score (b). Some animals were dosed with anti-mouse IL-6 antibody 10mg/kg s.c. once a week from one day prior to sensitisation. Data presented as mean ± s.e.m, n = 15 per group, *** $p<0.001$ statistically significant from indicated control. The number indicates percent inhibition of CIA with anti-IL-6 antibody treatment.

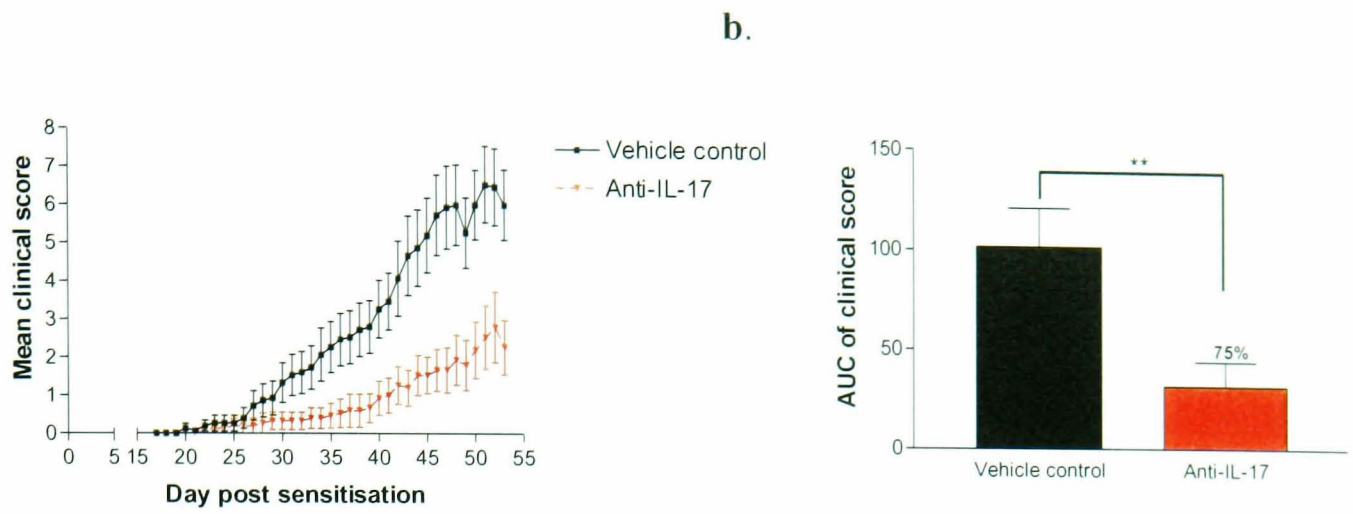


Figure 3.9 Effect of prophylactic dosing with anti-IL-17 antibody on collagen-induced arthritis.

Male DBA/1 mice were sensitised by intradermal injection of 100μg collagen II (CII) in 0.1M acetic acid and complete Freund's adjuvant at the base of the tail, 14 days post-sensitisation animals were boosted with 100μg CII in incomplete Freund's adjuvant. Paws were scored daily for clinical signs of arthritis on a 0-3 scale taking into account swelling of the wrist/ankle, pad and digits. Data presented as mean clinical score (a) and AUC of the clinical score (b). Some animals were dosed with anti-mouse IL-17 antibody 10mg/kg s.c. once a week from one day prior to sensitisation. Data presented as mean ± s.e.m, n = 15 per group, ** $p<0.01$ statistically significant from indicated control. The number indicates percent inhibition of CIA with anti-IL-17 antibody treatment.

3.4 Discussion

In this chapter a panel of drugs with different mechanisms of action were assessed in the CIA model and the results are summarised in Table 3.1 below.

Drug	**CIA**
Anti-$CD4^+$	+++ **(100)**
Anti-$CD8^+$	- **(19)**
Leflunomide	++ **(68)**
Anti-CD40L	+++ **(81)**
Anti-LFA-1	- **(38)**
Anti-TNFα	+++ **(82)**
Anti-IL-1β	+++ **(100)**
Anti-IL-6	+++ **(87)**
Anti-IL-17	+++ **(75)**

Table 3.1 Summary of drug effects on CIA.

Note: *The data is semi quantitative where + represents a low, ++ represents a medium and +++ represents a high degree of suppression, all of which are statistically significant as compared to controls.*

- represents no statistically significant change from control.

The numbers in brackets represent the percent reduction in the AUC of the clinical score as compared to controls.

Although the CIA model is widely used to assess the effects of anti-arthritic drugs it is poorly characterised in the literature. Results presented in this chapter demonstrate that a depleting mAb directed against $CD4^+$ T cells causes a complete abrogation in CIA. This confirms previous reports by Chu and Londei (1996) showing that

depleting and non-depleting Abs directed against CD4 inhibit CIA. However, in these studies, some mice showed signs of disease after treatment. This may be explained as they only treated mice for 11 days with Ab, thus the effects of treatment may have worn off allowing the re-population of $CD4^+$ T cells. The current study demonstrates that continual treatment is required to completely prevent signs of arthritis. From the data reported here and in the literature it is evident that $CD4^+$ T cells play a crucial role in the development of arthritis in this model.

In contrast, it is clear that $CD8^+$ T cells do not contribute to disease pathogenesis in the CIA model, as depletion of this cell population failed to inhibit arthritis. The majority of research conducted on $CD8^+$ T cells in CIA has been done in CD8 deficient mice. These mice have been reported to have a 5% reduction in the total number of T cells as compared to wild type mice and 80% of these cells are $CD4^+$ (Taneja *et al.*, 2002). The use of CD8 deficient mice has given rise to conflicting data. Taneja *et al.* (2002) demonstrated that CIA develops with increased disease severity in $CD8^+$ deficient mice, suggesting $CD8^+$ T cells have a regulatory role to play in disease. Whereas Tada *et al.* (1996) showed that $CD8^+$ T cells are involved in the initiation of disease, while Ehinger *et al.* (2001) reported CD8 deficiency had no effect on CIA. The variability between these studies may be due to different strains of mice being used to obtain $CD8^+$ deficiency. In support of a regulatory role for $CD8^+$ T cells in CIA Kadowaki *et al.* (1994) showed, in a transfer model of CIA, that depleting spleen cell cultures of $CD8^+$ T cells prior to transfer into SCID recipient mice enhanced the onset of arthritis. Data in this chapter confirms work conducted by Ehinger *et al.* (2001) and showed that in DBA/1 mice, the most commonly used strain to study CIA in, $CD8^+$ T cells are not important in the development of arthritis.

The results in this chapter clearly demonstrate that the T cell subset that drives disease in the CIA model is $CD4^+$.

To further investigate the underlying immunological processes in the CIA model mice were treated with anti-CD40L and anti-LFA-1 mAbs. Administration of anti-CD40L caused a statistically significant inhibition in the clinical signs of CIA. This demonstrates that the association between CD40L on T cells and its ligand CD40 on B cells is important in the immune response against CII. These data confirm the reported role of CD40L in CIA (Durie *et al.*, 1993). Durie *et al.* also showed that the production of anti-CII Abs was attenuated after CD40L blockade. The data reported here, as well as the literature, indicate the importance of T cell-B cell interactions in disease development and anti-CII Ab production in the CIA model.

Anti-LFA-1 treatment failed to inhibit signs of arthritis in the CIA model, which was surprising considering the fundamental importance of T cell-APC contact in antigen recognition. These data suggest that LFA-1 is not essential in the development of arthritis in this model. This finding is in contrast to work conducted by Kakimoto *et al.* (1992) who demonstrated that mAbs against LFA-1 and its ligand ICAM are capable of inhibiting CIA. However, the suppression of disease reported by Kakimoto *et al.* was not profound and statistical significance was only detected towards the end of the experiment. If these data were expressed as AUC of the clinical score, which would take into account the overall disease severity, this statistically significant effect may be lost giving a similar result as reported in the current study.

To show the CIA model responds to drugs used in the treatment of RA, leflunomide, a DMARD that inhibits lymphocyte proliferation, was assessed. Leflunomide caused

a statistically significant inhibition in clinical signs of CIA, something that has not been well documented in the past. These data validate CIA and demonstrate it is a clinically relevant model to assess anti-arthritic drugs in. These data also confirm previous reports of the anti-arthritic effect of leflunomide and its associated metabolites in other animal models of arthritis (Thoss *et al.*, 1996; Schorlemmer and Schleyerbach, 1998). Lymphocyte proliferation is therefore an important process in the immune response to CII and development of arthritis.

Anti-TNFα therapy has quickly become the gold standard in the treatment of RA, with biologics directed against IL-1 and IL-6 also being effective. In this chapter mAbs that target TNFα, IL-1β and IL-6 were shown to cause a statistically significant reduction in clinical signs of arthritis which further validates CIA as a relevant pre-clinical model of RA. In addition, these findings confirm previous reports showing that anti-TNFα treatment inhibits CIA (Williams *et al.*, 1992). Furthermore, in the current study anti-TNFα treatment caused a delay in disease onset, something that was not identified in the Williams *et al.* paper. Other workers have shown that anti-IL-1β treatment, initiated three days post-sensitisation, prevents the development of CIA (Geiger *et al.*, 1993). The data presented here confirms these findings and demonstrates that there is no additional benefit from dosing prior to sensitisation.

The work conducted with anti-IL-6 in this chapter has shown for the first time that a mAb against IL-6 effectively inhibits clinical signs of arthritis in the murine CIA model. Blockade of the IL-6 axis has previously been reported to attenuate CIA. However, this was conducted using a mAb against the IL-6 receptor (Takagi *et al.*, 1998) rather than IL-6 itself. The present data therefore confirms a role for IL-6 in disease pathogenesis and demonstrates that an alternative IL-6 blocking strategy is

effective in the CIA model. Overall these data demonstrate that the anti-cytokine mAbs have comparable anti-arthritic activity in CIA and demonstrate that the model is TNFα, IL-1β and IL-6 dependent.

The use of a mAb directed against the novel cytokine, IL-17, caused a statistically significant reduction in the signs of arthritis clearly demonstrating a role for IL-17 in CIA. This finding confirms reports showing that inhibition of IL-17 with an IL-17 receptor Fc (IL-17R:Fc) fusion protein, polyclonal anti-IL-17 Ab positive serum and an anti-IL-17 mAb reduce clinical signs of CIA (Lubberts *et al.*, 2001, Lubberts *et al.*, 2004 and Chu *et al.*, 2007 respectively). However, these were all administered either at the time of boost or after the onset of disease. The present study therefore appears to be the first to demonstrate an attenuation of CIA using a mAb dosed prior to sensitisation in the murine CIA model. The degree of inhibition seen with anti-IL-17 was comparable to that of the other anti-arthritic drugs suggesting IL-17 has potential as a therapeutic target.

This chapter has demonstrated that the immune response underlying CIA is $CD4^+$ T cell driven. Furthermore, it has been shown that T cell–B cell interactions and proliferation are essential in the development of arthritis. The lack of effect seen with anti-$CD8^+$ has confirmed that $CD8^+$ T cells do not play a role in this disease. In addition, it has become evident that the association between LFA-1 and ICAM-1 is not vital for T cell antigen recognition and hence the initiation of immunity. Anti-$CD8^+$ and anti-LFA-1 will therefore be used as negative controls throughout this thesis and it will be interesting to see if the pre-arthritic models and assays are capable of predicting their lack of efficacy in CIA.

Assessment of leflunomide and mAbs against clinically relevant pro-inflammatory cytokines has validated the CIA model in anti-arthritic drug discovery. Moreover, these drugs have demonstrated a role for TNF-α, IL-1β and IL-6 in disease pathogenesis. Additionally, a role for IL-17 in disease has been demonstrated and the clinical relevance of the CIA model suggests an anti-IL-17 therapeutic may have utility in the clinic.

The anti-arthritic drugs assessed in this chapter have varying mechanisms of action. However, they all caused a similar degree of disease suppression (Table 3.1) which highlights the point that the CIA model is not capable of discriminating between anti-arthritic drugs. In the following chapters, early readouts of CII immunity were established. The drugs used above were then assessed in these pre-arthritic models and assays to determine if they are predictive of anti-arthritic drug effects. Furthermore, the results from these studies may help discriminate between drugs based on mechanism of action.

Chapter four

Short-term models of CII immunity

4.1 Introduction

The pathogenesis of CIA is considered to be dependent on both humoral and cellular components of the immune system (Seki *et al.*, 1988) and data presented in Chapter 3 supports this. Evidence in the literature has demonstrated that humoral and cellular readouts of CII immunity, such as anti-CII Ab titres (Williams *et al.*, 1998), CII-induced hypersensitivity (Farmer *et al.*, 1986) and CII stimulated cell proliferation (Mauri *et al.*, 1996) can be detected in pre-arthritic mice. These readouts have the potential to be used to assess drug effects on CII immunity prior to disease onset and may be predictive of anti-arthritic drug activity in the CIA model.

To be able to investigate this, models and assays of CII immunity were set up and studied. In addition to these readouts the acute phase protein, serum amyloid A (SAA), which is released from the liver during inflammation, was assessed in the CIA model. It has been demonstrated that SAA is present in arthritic mice (Palmer *et al.*, 2003) and therefore has the potential to act as a marker of inflammation.

In this chapter anti-CII IgG1 and anti-CII IgG2a production was assessed in CII sensitised mice over the course of a CIA study. The relationship between pre-arthritic anti-CII Ab production and subsequent disease severity was then investigated. SAA levels were also assessed over the time course of CIA to see if this acute phase protein can be detected in CII sensitised mice prior to disease onset. These studies were used to identify a time point in the pre-arthritic phase when CII immunity could be detected. A CII-induced hypersensitivity model and a CII stimulated $CD4^+$ T cell thymidine incorporation assay were then set up in this phase and the conditions required to produce robust and reproducible readouts were investigated.

4.2 Aim

The aim of this chapter was to develop robust short-term models of CII immunity in pre-arthritic mice that are suitable for studying the immunomodulatory properties of drugs.

4.3 Results

In these experiments data is presented as the mean per group. CIA is presented as the clinical score on a daily basis and AUC of the clinical score. The assessment of anti-CII IgG1/IgG2a was conducted by ELISA with a colorimetric end point which was measured as absorbance (optical density, OD) at 450nm wavelength. SAA levels were calculated as μg/mL in serum by ELISA. Hypersensitivity to CII was assessed by measuring the change in ear thickness, in millimetres (mm), between normal and sensitised mice. In CII stimulated $CD4^+$ T cell assays thymidine incorporation was quantified as counts per minute (cpm). Statistical analysis of data was conducted either by Mann Whitney test or one-way ANOVA with Bonferroni as the post-test and $p<0.05$ was considered statistically significant.

4.3.1 Anti-CII IgG1 and IgG2a production in CIA

To identify when anti-CII IgG1 and anti-CII IgG2a production can be detected, a time course of CIA was set up (Figure 4.1). Normal mice or mice sensitised to CFA produced no anti-CII Ab of either isotype throughout the study. Mice sensitised to CII/CFA showed no signs of anti-CII IgG1 or anti-CII IgG2a production 7 days post-sensitisation. However, on Day 14 post-sensitisation, anti-CII IgG1 was detected with a mean absorbance at 450nm of 0.45 ± 0.13 OD, this steadily increased over time reaching a peak on Day 42 of 2.03 ± 0.35 OD (Figure 4.1a), before decreasing on Day 49. Following a similar pattern, anti-CII IgG2a was detected on Day 14 post-sensitisation with a mean absorbance of 0.43 ± 0.14 OD, this increased to 1.90 ± 0.41 OD on Day 42 with a decrease on Day 49 (Figure 4.1b). In this study signs of arthritis were seen from Day 28 onwards where the mean clinical score increased from 1.80 ± 1.11 to 5.20 ± 1.66 on Day 42 and then decreased to 3.60 ± 0.40 on Day 49 (data not

shown). The increase and then decrease in disease appears to mirror the production of anti-CII Ab isotypes at the time points assessed.

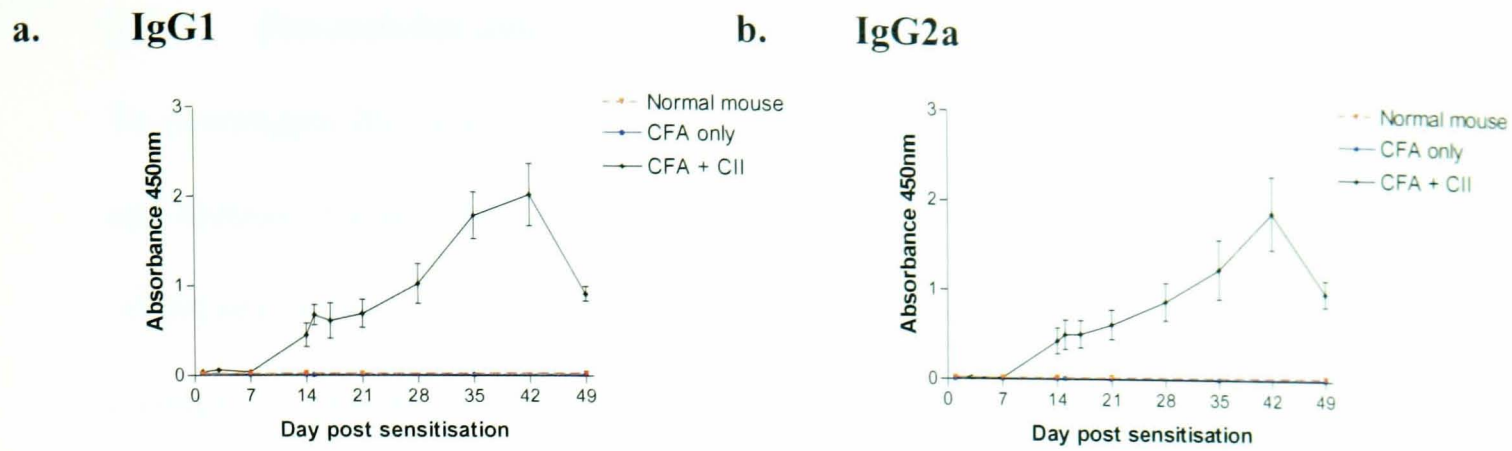


Figure 4.1 Anti-collagen II antibodies in collagen-induced arthritis.

Anti-collagen II IgG1 and IgG2a production was assessed in normal male DBA/1 mice and in mice sensitised at the base of the tail with either complete Freund's adjuvant (CFA) or 100µg collagen II (CII) in CFA. Blood was taken via cardiac puncture from 4-5 mice on Day 1, 3, 7 and 14 post-sensitisation and serum was separated. On Day 14, mice sensitised to CFA received a boost injection of incomplete Freund's adjuvant (IFA) and mice sensitised to CFA/CII were boosted with 100µg CII in IFA. Further serum samples were taken on Day 15, 17, 21, 28, 35, 42 and 49 from 4-5 mice per time point. Serum samples were collected and stored at -70°C for later analysis. Anti-CII IgG1 (a) and IgG2a (b) production was detected in serum samples by ELISA as described in Chapter 2. Data is presented as mean ± s.e.m.

4.3.2 Pre-arthritic anti-CII IgG1 and IgG2a as markers of CIA

To investigate the relationship between pre-arthritic anti-CII IgG1/IgG2a production and disease, blood samples were taken via the tail in a CIA study 14 days post-CII sensitisation and serum was separated. Signs of arthritis were seen from Day 20 onwards and increased over time, resulting in a mean clinical score of 5.44 ± 1.91 on Day 44 (Figure 4.2a). Serum from CII sensitised pre-arthritic mice showed a statistically significant increase in both anti-CII IgG1 and IgG2a production as compared to normal mice (Figure 4.2b). Plotting individual absorbance levels of anti-CII IgG1 (Figure 4.2c) and anti-CII IgG2a (Figure 4.2d) against the corresponding area under the curve (AUC) of the clinical score, obtained over a 44 day period, resulted in a statistically significant correlation.

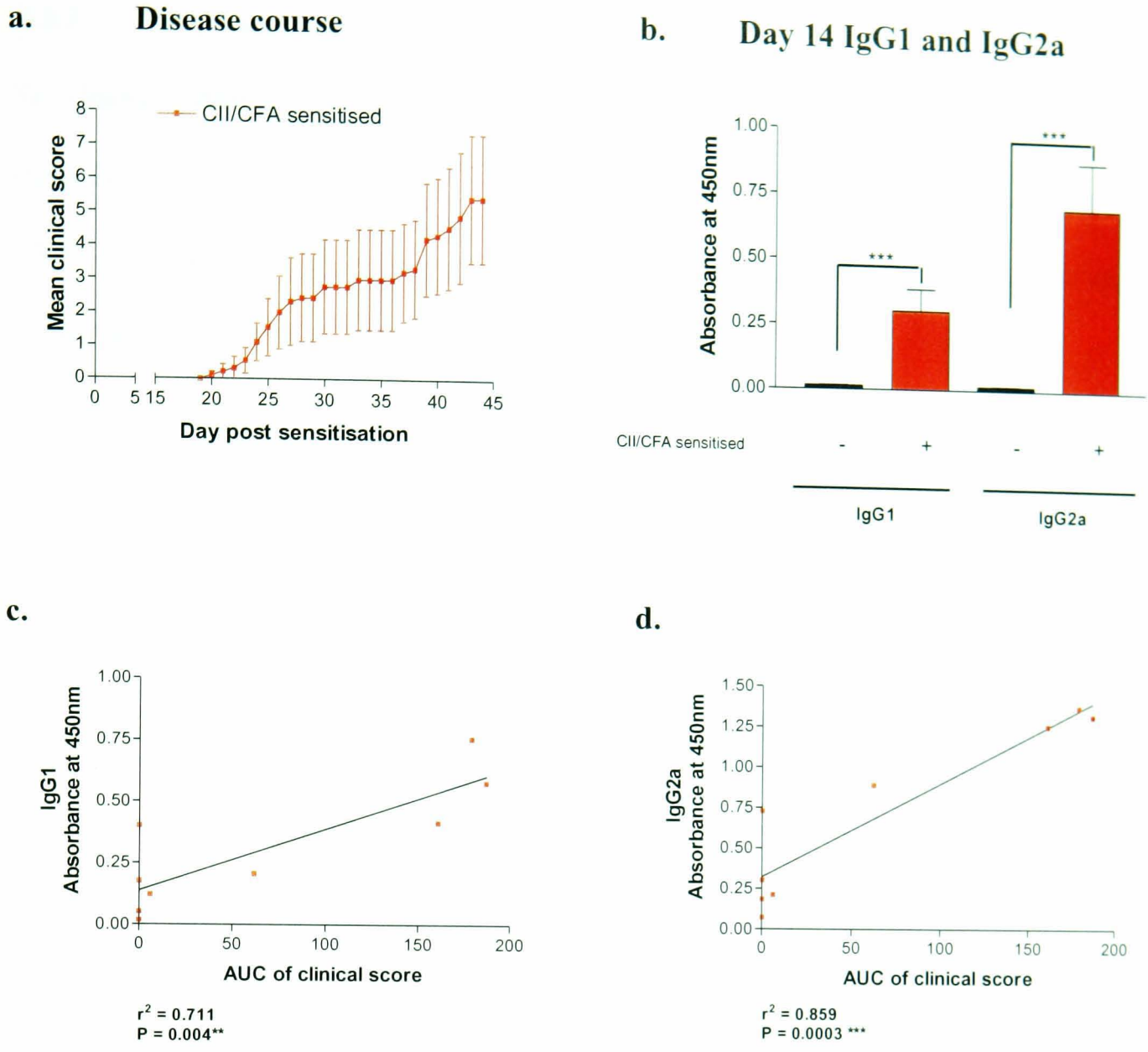


Figure 4.2 Pre-arthritic anti-chick collagen II antibodies and collagen-induced arthritis.

Male DBA/1 mice were immunised at the base of the tail with 100μg collagen II (CII) in complete Freund's adjuvant (CFA). On Day 14 post-sensitisation blood samples were taken via the tail and serum was separated and stored at -70°C. Mice then received a boost injection of 100μg CII in incomplete Freund's adjuvant. Serum samples were also taken from 5 normal mice at this time point. Paws were scored daily for clinical signs of arthritis on a 0-3 scale taking into account swelling of the wrist/ankle, pad and digits. Data is presented as mean clinical score (a). Anti-CII IgG1 and anti-CII IgG2a (b) production was detected in Day 14 serum samples by ELISA as described in Chapter 2. The area under the curve (AUC) of the clinical score was calculated and correlated with Day 14 anti-CII IgG1 (c) and anti-CII IgG2a (d) production. Data presented as mean ± s.e.m, n = 9 CFA/CII sensitised mice and n = 5 normal mice, ** $P<0.01$ and *** $P<0.001$ statistically significant.

4.3.3 Serum amyloid A (SAA) levels in CIA

To identify when the acute phase protein SAA is produced in CIA, serum samples from the time course described above were analysed. Figure 4.3 shows the level of SAA detected from serum of normal mice, mice sensitised to CFA only and mice sensitised to CII/CFA.

Normal mice showed a low level of circulating SAA, 70.28 ± 30.61 μg/mL, on Day 49. Sensitisation with either CFA or CII/CFA caused a similar increase in SAA levels to 4240.00 ± 1799.00 and 5719.00 ± 457.00 μg/mL respectively 1 day post-sensitisation. From Day 1 to Day 14 SAA levels in CFA and CII/CFA sensitised mice decreased back to levels seen in normal mice (77.07 ± 37.29 and 30.71 ± 8.20 μg/mL respectively).

On Day 14, CFA sensitised mice were given an injection of IFA and CII/CFA mice received an injection of CII/IFA. SAA levels in serum samples taken 1 day after this (Day 15) increased in response to IFA, 943.30 ± 504.70 μg/mL and CII/IFA, 3424.00 ± 1670.00 μg/mL, with no difference being seen between the two groups. From Day 15 to Day 35 SAA levels in CFA sensitised mice decreased back to levels consistent with normal mice, 23.93 ± 6.14 μg/mL and stayed low until termination of the experiment on Day 49. CII/CFA sensitised mice also showed a decrease in SAA levels up to Day 21, 110.50 ± 15.44 μg/mL. Then, in contrast to CFA sensitised mice, there was a steady increase in SAA levels from Day 28 onwards, reaching a statistically significant increase of 2982.00 ± 1124.00 μg/mL on Day 49 as compared to 21.25 ± 9.31 μg/mL in CFA sensitised mice. This increase in SAA coincided with the development of arthritis in the CII/CFA group (data not shown), whereas no arthritis was detected in the CFA group.

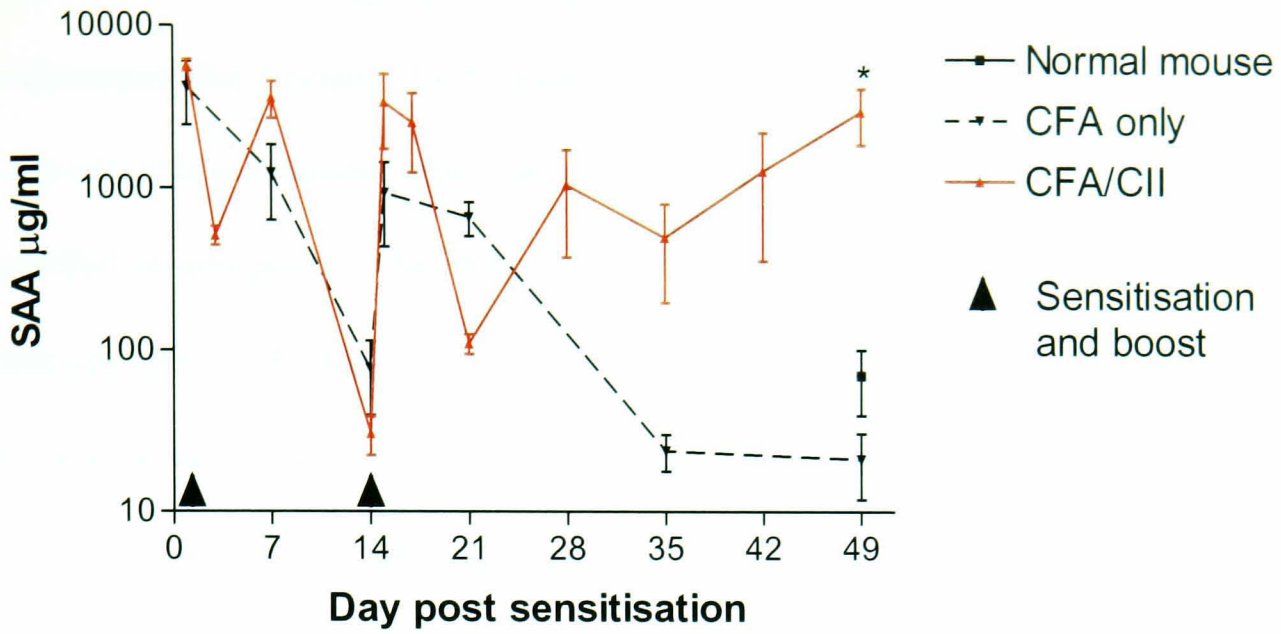


Figure 4.3 Serum amyloid A levels in collagen-induced arthritis.

Male DBA/1 mice were sensitised at the base of the tail with either complete Freund's adjuvant (CFA) or 100µg collagen II (CII) in CFA. Blood samples were taken via cardiac puncture from 4-5 mice on Day 1, 3, 7 and 14 post-sensitisation and serum was separated. On Day 14, mice sensitised to CFA received a boost injection of incomplete Freund's adjuvant (IFA) and mice sensitised to CFA/CII were boosted with 100µg CII in IFA. Further serum samples were taken on Day 15, 17, 21, 28, 35, 42 and 49 from 4-5 mice per time point. Serum was also separated from the blood of 5 normal mice on Day 49. Serum samples were stored at -70°C for later analysis. Serum amyloid A levels were detected in serum samples using an ELISA kit. Data presented as mean ± s.e.m. * $p<0.05$ statistically significant from CFA control.

4.3.4 CII-induced hypersensitivity in the ear

To determine the amount of CII needed to produce a robust hypersensitivity reaction in the ear, a dose response was conducted (Figure 4.4). The ear thickness of mice was measured in mm prior to the initiation of a hypersensitivity response (0 hours). Mice sensitised to CII 14 days earlier were injected with either 2, 6 or 20μg of CII in the ear. Normal mice were used as a control and received an injection of 20μg CII in the ear. Ear thickness was then measured at 6 and 24 hours post-CII challenge. The change in ear thickness at 6 and 24 hours as compared to 0 hours was calculated. At 6 hours post-challenge normal mice showed an increase in ear thickness of 0.07 ± 0.01 mm in response to 20μg CII. CII sensitised mice challenged in the ear with 2, 6 or 20μg CII produced a statistically significant increase in ear thickness of 0.21 ± 0.01, 0.22 ± 0.02 and 0.25 ± 0.01 mm respectively as compared to normal mice. At 24 hours post-CII challenge normal mice showed an increase in ear thickness of 0.05 ± 0.01 mm. The ear thickness in CII sensitised mice challenged with 6 or 20μg CII at this time point increased to 0.17 ± 0.02 and 0.23 ± 0.02 mm respectively and was statistically significant as compared to normal mice. However, 2μg CII failed to produce a significant increase in ear thickness as compared to normal mice (0.12 ± 0.01 mm). The statistically significant increase in ear swelling seen in CII sensitised mice as compared to normal mice therefore reflects CII hypersensitivity.

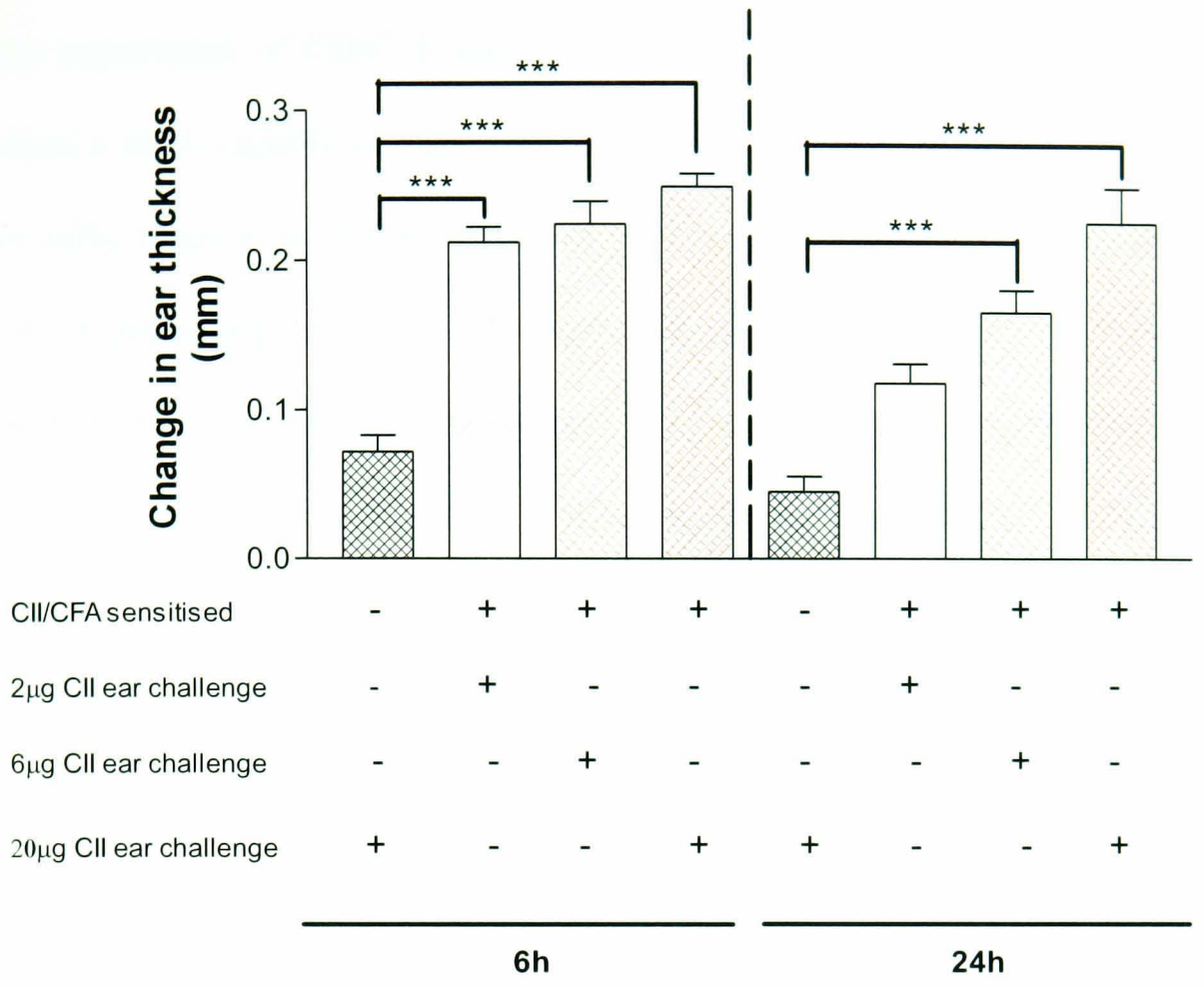


Figure 4.4 Collagen II hypersensitivity in the mouse ear.

Collagen II (CII) hypersensitivity was assessed in normal male DBA/1 mice or in mice 14 days after sensitisation with CII in complete Freund's adjuvant. Animals were challenged with either 2, 6 or 20μg CII in 25μL phosphate buffered saline (PBS) injected into the right ear. The left ear was injected with PBS as a control. Ear thickness was measured in mm using callipers at 6 and 24 hours post-challenge. Data presented as mean ± s.e.m, n = 10 per group, *** $p<0.001$ statistically significant from indicated control.

4.3.5 CII stimulated CD4$^+$ T cell thymidine incorporation assay

The importance of CD4$^+$ T cells in the CIA model has been shown in Chapter 3, where a mAb capable of depleting CD4$^+$ T cells caused total abrogation of disease. An early readout of cellular immunity to CII would therefore be of great value, not only in predicting the effects of anti-arthritic drugs, but also when investigating their mechanisms of action. To address this, a CII specific *ex vivo* CD4$^+$ T cell thymidine incorporation assay was set up. This assay used CD4$^+$ T cells isolated from pre-arthritic mice taken 14 days post-CII sensitisation and antigen presenting cells (APCs) from normal mice. This time point was chosen as it coincides with the detection of anti-CII Ab production and CII-induced hypersensitivity. CII at a concentration of 50μg/mL was added *in vitro* to some cells as it has previously been shown to cause an increase in thymidine incorporation into cells from CII sensitised mice (Mauri *et al.*, 1996). To establish robust and reproducible conditions for CII stimulated thymidine incorporation, differing concentrations of CD4$^+$ T cells and APCs were investigated (Figure 4.5). Thymidine incorporation was detected as counts per minute (cpm). CD4$^+$ T cells were plated out at either 2.5 x 10^4 or 2.5 x 10^5 cells per well, with 2.5 x 10^4, 2.5 x 10^5 or 1 x 10^6 APCs in the presence or absence of CII. CII stimulation of CD4$^+$ T cells at a concentration of 2.5 x 10^4 in combination with 2.5 x 10^4 or 2.5 x 10^5 APCs increased thymidine incorporation above that of non-stimulated cells by 5474 and 17272 cpm respectively. Increasing the concentration of APCs to 1 x 10^6 in culture with these CD4$^+$ T cells did not increase thymidine incorporation further (1433 cpm) which suggests it is the concentration of CD4$^+$ T cells that are the limiting factor. This was confirmed by increasing the number of CD4$^+$ T cells to 2.5 x 10^5 in combination with 2.5 x 10^4, 2.5 x 10^5 or 1 x 10^6 APCs which resulted in a much greater increase in CII stimulated thymidine incorporation

(97270, 90738 and 164922 cpm respectively). From these data it was decided to use 2.5 x 10^5 $CD4^+$ T cells and 1 x 10^6 APCs in all subsequent experiments as this combination produced the greatest increase in CII stimulated thymidine incorporation.

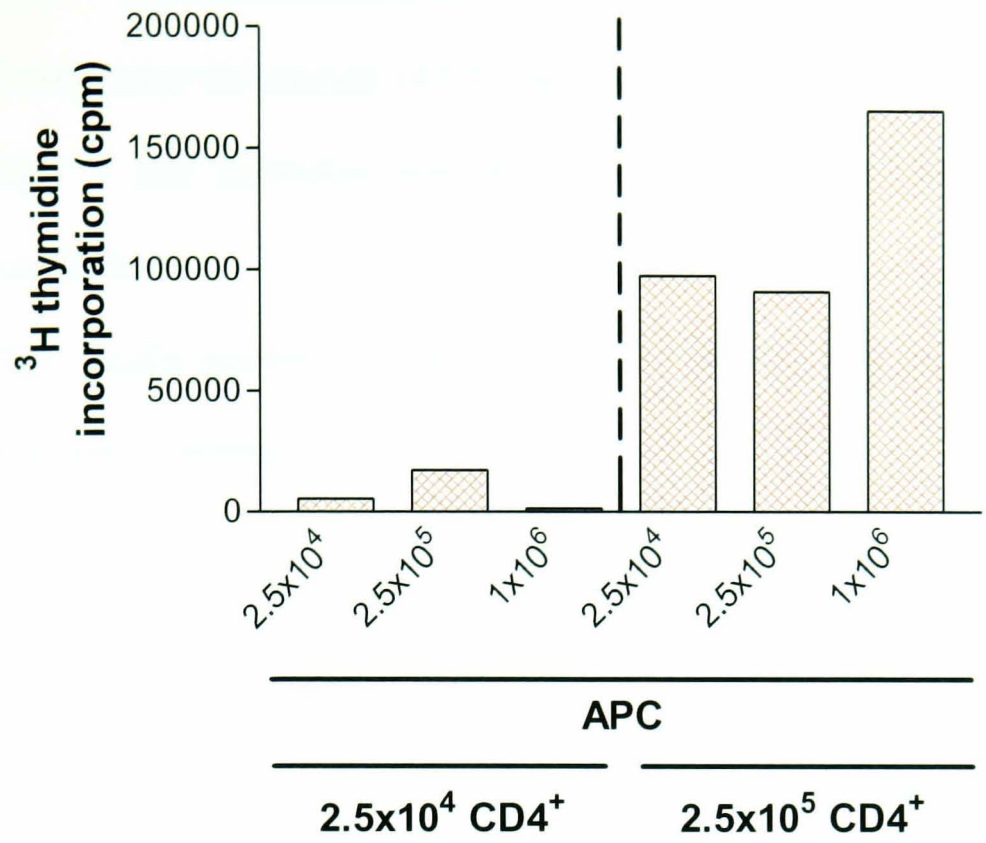


Figure 4.5 CD4$^+$ T cell and APC concentrations in the *ex vivo* thymidine incorporation assay.

CD4$^+$ T cells were prepared from inguinal lymph nodes of DBA/1 mice 14 days after sensitisation with collagen II (CII) in complete Freund's adjuvant (CFA). Cells were plated out at 2.5 x 10^4 or 2.5 x 10^5 CD4$^+$ T cells together with 2.5 x 10^4, 2.5 x 10^5 or 1 x 10^6 mitomycin C treated antigen presenting cells obtained from normal mice spleens. In some wells, cells were stimulated with 50μg/mL denatured CII. Cells were cultured for 72 hours. Tritiated thymidine was added to the wells for the last 6 hours of culture and thymidine incorporation was assessed as counts per minute (cpm). The graph shows the change in cpm after *in vitro* CII stimulation of cells from CII/CFA sensitised mice as compared to non-stimulated cells. Lymph nodes were pooled from n = 10 mice and cells were plated out in replicates of 5.

4.3.6 CII concentration in the CD4$^+$ T cell thymidine incorporation assay

To determine the amount of CII required to stimulate a robust response in the 14 day CD4$^+$ T cell thymidine incorporation assay, various concentrations of CII were investigated.

CD4$^+$ T cells isolated from normal mice, CFA sensitised mice and CII/CFA sensitised mice were cultured with APCs from normal mice in the presence or absence of 0.5, 5 or 50μg/mL CII. CD4$^+$ T cells isolated from normal mice did not respond to 50μg/mL CII, as indicated by a lack of increase in cpm between non-stimulated (4054 ± 264 cpm) and CII stimulated (4092 ± 229 cpm) cells (Figure 4.6). CD4$^+$ T cells isolated from mice that had received CFA sensitisation gave a mean 10920 ± 777 cpm. When the same cells were stimulated with 50μg/mL CII there was no increase in cpm (11048 ± 463), indicating a lack of additional thymidine incorporation in response to CII. CD4$^+$ T cells from CII sensitised mice cultured without CII showed a background of 15315 ± 953 cpm and in a concentration dependent manner *in vitro* CII stimulation of these cells caused a statistically significant increase to 24411 ± 378, 34933 ± 2157 and 46620 ± 2227 cpm at 0.5, 5 and 50μg/mL CII respectively.

These data indicate that 50μg/mL CII stimulates the greatest increase in thymidine incorporation therefore all subsequent CD4$^+$ T cell thymidine incorporation assays used this concentration of CII. Results reported in this study along with those in Figure 4.5 indicate that CD4$^+$ T cells at a concentration of 2.5 x 10^5 and APCs at a concentration of 1 x 10^6 in the presence of 50μg/mL CII give a robust and reproducible response. In addition, there appears to be a trend towards increased incorporation of thymidine between non-stimulated cells isolated from normal mice and non-stimulated cells isolated from both CFA and CII/CFA sensitised mice.

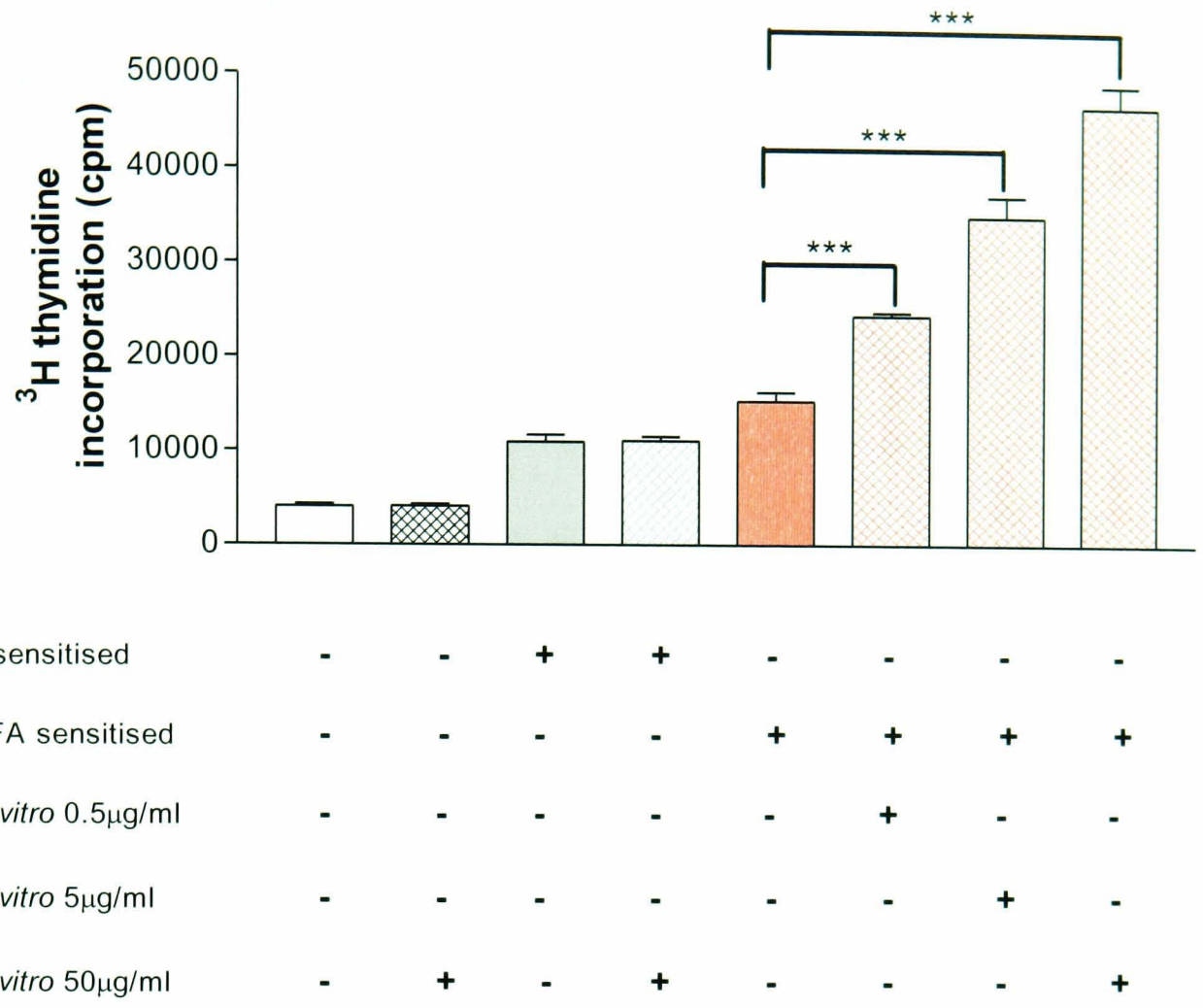


Figure 4.6 Collagen II stimulation in the thymidine incorporation assay.

$CD4^+$ T cells were prepared from inguinal lymph nodes of normal male DBA/1 mice or from mice 14 days after sensitisation with either complete Freund's adjuvant (CFA) or collagen II (CII) in CFA. Cells were plated out at 2.5 x 10^5 $CD4^+$ T cells together with 1 x 10^6 mitomycin C treated antigen presenting cells obtained from normal spleens. In some wells, cells were stimulated with either 0.5, 5 or 50μg/mL denatured CII. Cells were cultured for 72 hours. Tritiated thymidine was added to the wells for the last 6 hours of culture and thymidine incorporation was assessed as counts per minute (cpm). Lymph nodes were pooled from n = 5-10 mice per group and cells were plated out in replicates of 5. Data presented as mean ± s.e.m, *** $p<0.001$ statistically significant from indicated control.

4.3.7 Time course of CII stimulated CD4$^+$ T cell thymidine incorporation

Using the conditions outlined above, a time course of CII stimulated CD4$^+$ T cell thymidine incorporation was conducted (Figure 4.7). CD4$^+$ T cells from normal mice, CFA sensitised mice and CII/CFA sensitised mice were cultured for 24, 48, 72 and 96 hours with or without CII stimulation. Cells from normal and CFA sensitised mice showed no signs of increased thymidine incorporation in response to CII stimulation at any time point. Over the time course studied there was, however, a trend towards increased thymidine incorporation in these cells. When CD4$^+$ T cells from CII/CFA sensitised mice were cultured without CII, no increased thymidine incorporation above that of control cells was seen at any time point. However, when these cells were cultured with CII, increased thymidine incorporation was evident at 24, 48, 72 and 96 hours. This gave a statistically significant increase in cpm, as compared to non-stimulated cells, from 7060 ± 252 to 14554 ± 644, 22080 ± 317 to 56114 ± 7690, 48032 ± 4483 to 204882 ± 3947 and 76072 ± 9137 to 248296 ± 11843 cpm respectively. As shown, this CII stimulated thymidine incorporation increased over time from 14554 ± 644 cpm at 24 hours to 248296 ± 11843 cpm at 96 hours, with the greatest difference between non-stimulated and CII stimulated cells being detected at 72 and 96 hours of culture.

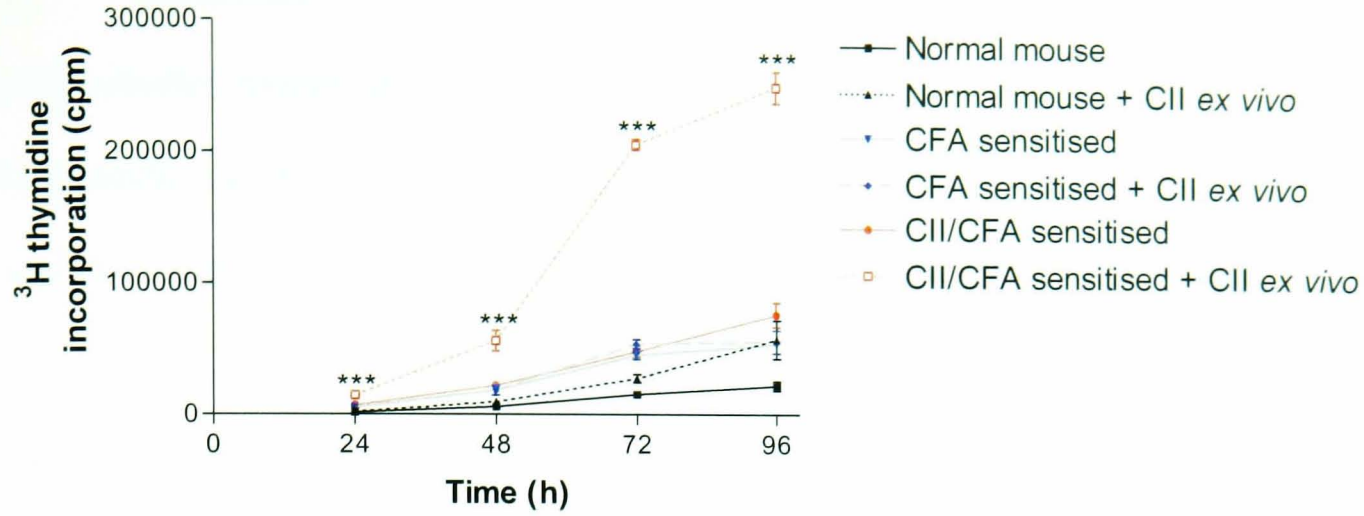


Figure 4.7 *Ex vivo* thymidine incorporation into CD4$^+$ T cells over time.

CD4$^+$ T cells were prepared from inguinal lymph nodes of normal male DBA/1 mice or from mice 14 days after sensitisation with either complete Freund's adjuvant (CFA) or collagen II (CII) in CFA. Cells were plated out at 2.5 x 10^5 CD4$^+$ T cells together with 1 x 10^6 mitomycin C treated antigen presenting cells obtained from normal spleens. In some wells, cells were stimulated with 50μg/mL denatured CII. Tritiated thymidine was added to the wells for the last 6 hours of culture and thymidine incorporation was assessed as counts per minute (cpm). In this experiment cells were cultured for 24, 48, 72 and 96 hours. Lymph nodes were pooled from n = 5-10 mice per group and cells were plated out in replicates of 5. Data presented as mean ± s.e.m, *** $p<0.001$ between CII/CFA sensitised and CII/CFA sensitised + *in vitro* CII.

4.4 Discussion

In this chapter, models and assays were developed in pre-arthritic mice to assess both the humoral and cellular response of the immune system to CII. From these data it is clear that a CII specific humoral response can be seen in pre-arthritic mice as early as 14 days post-CII sensitisation. This is indicated by a statistically significant increase in anti-CII IgG1 and anti-CII IgG2a Ab production. The production of these anti-CII isotypes on Day 14 post-sensitisation showed a statistically significant correlation to the AUC of the clinical score. This indicates that the strength of the humoral response to CII in the pre-arthritic phase is directly related to the severity of arthritis that a mouse will develop. Thus, this 14 day readout of CII immunity has the potential to predict disease severity in the CIA model. These data confirm previous findings reported by Williams *et al.* (1998) who showed a similar correlation between pre-arthritic anti-CII Ab production and disease severity. However, their study used serum 21 days post-sensitisation, which is around the time of disease onset and they only looked at whole anti-CII IgG levels. The data presented here therefore extends these findings indicating that earlier detection of anti-CII Abs is possible and gives the same correlation as samples taken on Day 21.

Furthermore, it has been demonstrated that both anti-CII IgG1 and anti-CII IgG2a Ab isotypes correlate to disease severity. The detection of anti-CII IgG1 and anti-CII IgG2a isotypes also gives an indication of Th2 and Th1 cell responses respectively therefore suggesting that these cells are present. This is something that could not be assessed by measuring IgG in the Williams *et al.* study. Assessing these anti-CII Ab isotypes has the potential to assess drug effects on $CD4^+$ T cell subsets and may help discriminate between drug mechanisms of action.

SAA levels monitored during the time course of CIA suggest it is the adjuvants, CFA or IFA, that stimulate the production of this acute phase protein early on in the immune response (Day 1-21) and not CII itself. However, the adjuvant induced SAA effect soon decreases to background levels while, in CII sensitised mice it increases, coinciding with signs of arthritis. This suggests that the inflammation in the paw is stimulating SAA release. Thus, SAA detected later in CIA appears to be a biomarker of joint inflammation. If SAA were to be utilised as a marker of disease it would have to be assessed in the arthritic phase and is therefore not suitable as an early readout. However, the early adjuvant induced SAA response could be utilised to assess the effect of a drug on general immunity. This could be done by taking serum from pre-arthritic mice between Day 1-7 post-sensitisation and identifying any inhibitory drug effect on SAA levels. Alternatively a CII challenge in the ear, as conducted in the hypersensitivity model, should elicit an SAA response. If this is the case then drug effects on SAA can be assessed at the same time as CII-induced hypersensitivity. This was investigated in Chapter 5. Monitoring SAA levels in this way may provide a general marker of drug activity on the immune system and could be predictive of anti-arthritic drug effects in the CIA model.

This chapter has demonstrated that a CII ear challenge in mice, 14 days post-CII sensitisation, causes an increase in ear thickness as compared to non-sensitised mice indicating that this response is immune driven. Measurements taken 6 hours post-CII ear challenge should give a readout of an immediate type hypersensitivity reaction. This is likely to be a type III IgG mediated hypersensitivity response as anti-CII IgG Abs are present in the circulation 14 days post-sensitisation. Measurements taken 24 hours post-challenge should reflect a T cell driven type IV DTH response and it is

evident from the $CD4^+$ T cell thymidine incorporation assay that CII specific cells are present. This CII-induced hypersensitivity model appears to be both Ab and T cell dependent.

CII caused an increase in ear thickness at 6 and 24 hours post-challenge in a dose dependent manner. It was decided from these data that an ear challenge of 20μg CII gave a robust hypersensitivity response and was used in all subsequent studies.

The data presented here confirms work conducted by Seki *et al.* (1988) who showed that a hypersensitivity response can be elicited in mice 14 days post-CII sensitisation. However, Seki *et al.* (1988) assessed hypersensitivity in the footpad. Thus, the current work has demonstrated that CII hypersensitivity can also be detected in the ear of CII sensitised mice at this time point. This readout has the potential to be incorporated into the CIA model as an early marker of drug activity. If this were the case, mice may continue on to develop CIA. In this instance measuring hypersensitivity in the footpad may not be appropriate as it may interfere with the clinical readout of the disease model. An ear measurement, as demonstrated here, would therefore be advantageous.

Hypersensitivity in these studies was conducted using chick CII as CII derived from mice gave rise to variable ear swelling responses between animals (historical data). These data indicate that the immune response to chick CII is stronger than that to mouse CII, which is not surprising as mice were sensitised to chick CII in both instances. However, this does suggest that a weaker immune response may occur in the joints of diseased mice in response to endogenous CII. This may lead to variability of drug effects between the hypersensitivity model and the CIA model.

The role of $CD4^+$ T cells in the pathogenesis of CIA was demonstrated in Chapter 3, where depletion of this cell population resulted in total inhibition of arthritis. These data confirmed similar findings in the literature (Chu and Londei, 1996) and demonstrate the importance of these cells in CII immunity, therefore making them a relevant cell population to study. In order to focus on this cellular component of CII immunity, a $CD4^+$ T cell thymidine incorporation assay was set up. The incorporation of thymidine into cells is a measure of the s-phase of the cell cycle where deoxyribonucleic acid is duplicated prior to cell division. This was therefore used as a surrogate marker of proliferation.

From the data presented here, it is evident that $CD4^+$ T cells isolated from lymph nodes of mice 14 days post-CII sensitisation proliferate in response to *in vitro* CII stimulation. This supports previous findings by Mauri *et al.* (1996) who showed lymph node cells from CII sensitised mice, in the pre-arthritic phase, proliferate in response to CII. However, in the Mauri *et al.* (1996) paper, it could not be determined if $CD4^+$ T cells were proliferating as they used a single cell suspension. By isolating $CD4^+$ T cells in the current study it is possible to have a readout of CII specific $CD4^+$ T cell proliferation. Considering the vital role that these cells play in CIA it would suggest drugs that inhibit this response are likely to have anti-arthritic activity. This relationship was investigated in the following chapters.

The data presented here suggest the number of $CD4^+$ T cells is crucial in this response, rather than the number of APCs, as $CD4^+$ T cells at 2.5×10^5 resulted in statistically significant increases in proliferation at all the APC concentrations investigated. Whereas a lower $CD4^+$ T cell concentration failed to produce such pronounced responses. The greatest increase in CII stimulated proliferation was seen using 2.5×10^5 $CD4^+$ T cells in combination with 1×10^6 APCs. Using these cell

concentrations, subsequent experiments identified that 50μg/mL CII added *in vitro* to cells from CII sensitised mice over a 72 hour culture period gave robust and reproducible proliferative responses. Culturing for a longer period of time (96 hours) was considered of no additional benefit. The culture conditions set out above were used in all $CD4^+$ T cell proliferation assays.

The data presented in this chapter also suggests that CFA and CII/CFA may cause an increase in background proliferation as compared to cells from normal mice and as this occurs in the absence of CII stimulation it appears to be a non-specific response.

Taken together these data show that CII specific humoral and cellular responses can be detected in pre-arthritic mice, 14 days post-CII sensitisation and represent promising markers of subsequent disease activity in CIA. However, SAA was not elevated above that of normal mice at this time point and appears to be induced by adjuvant rather than CII. Despite this, it may still be a potentially useful marker of non-specific inflammation.

This chapter has identified the conditions required to produce robust readouts of CII immunity in pre-arthritic mice. In the following chapters the analysis of anti-CII IgG1/IgG2a, SAA and CII hypersensitivity were incorporated into the same experiments. Whereas CII specific $CD4^+$ T cell proliferation was conducted separately as it was not known what effect a CII ear challenge would have on this readout. From the literature it does not appear that these early readouts have been used together to assess the anti-arthritic effects of drugs. In the following chapters the ability of these 14 day readouts to predict and differentiate anti-arthritic drug activity, as determined in Chapter 3, was investigated. The drugs under investigation in this thesis were used to characterise these models and assays. Furthermore, these readouts

were tested to see if they could be of value when assessing a novel drug target such as IL-17

Chapter five

Drug effects on CII-induced hypersensitivity and anti-CII antibody production

5.1 Introduction

Murine CIA is a long-term model which represents a considerable investment in time and resource. The ability to assess drug activity in short-term models that may be predictive of drug effects in CIA is therefore desirable. In this chapter drug effects on CII-induced hypersensitivity and anti-CII Ab production were assessed. Drugs were used at the same dose and frequency as in the CIA model, unless otherwise stated. Anti-$CD4^+$, anti-$CD8^+$, anti-CD40L and anti-LFA-1 mAbs were used to investigate the underlying immune processes involved in CII immunity. Leflunomide and mAbs that target the clinically relevant cytokines, TNFα, IL-1β and IL-6 were used to validate these readouts. These mAbs were also used to identify the cytokine dependency of the early immune response to CII.

Drug effects on these readouts of CII immunity were then compared to their anti-arthritic activity (Chapter 3) and the predictive nature of CII-induced hypersensitivity and anti-CII Ab production were assessed. The effect of anti-IL-17 was then investigated in these readouts to determine if they are capable of predicting its anti-arthritic activity and moreover to establish the role of IL-17 in CII immunity in pre-arthritic mice.

In addition to these readouts, SAA levels were assessed in the serum of mice at termination of the hypersensitivity model. If SAA levels were found to be elevated in CII sensitised mice, post-CII ear challenge, then this readout could also be assessed for its ability to predict anti-arthritic drug effects in CIA. This would give a general marker of drug activity on inflammation and may add to the potential value of this short-term model.

By assessing drug effects on CII-induced hypersensitivity at 6 and 24 hours post-challenge, anti-CII IgG1 and anti-CII IgG2a production and potentially the acute

phase response, it may even be possible to discriminate between drugs based on their mechanisms of action. The use of these readouts to predict and discriminate anti-arthritic drug activity in pre-arthritic mice has not been reported. Thus, the work in this chapter has the potential to identify a new early screening strategy for the assessment of novel anti-arthritic drugs.

5.2 Aim

The aim of this chapter was to characterise and validate the CII-induced hypersensitivity model and the anti-CII Ab response with the drugs utilised in Chapter 3 and to determine if these readouts can predict and discriminate anti-arthritic drug effects.

5.3 Results

In the following set of experiments data is presented as the mean per group. The assessment of CII-induced hypersensitivity was conducted by calculating the change in ear thickness, measured in millimetres (mm), between the left ear injected with PBS and the right ear injected with CII. A statistically significant increase in the change in ear thickness in mice sensitised to CII as compared to normal (non-sensitised) mice reflects a hypersensitivity reaction. The assessment of anti-CII IgG1/IgG2a was conducted by ELISA with a colorimetric end point which was measured as absorbance (optical density, OD) at 450nm wavelength. Drugs were assessed for their ability to reduce these readouts of CII immunity relative to vehicle treated controls. Statistical analysis of data was conducted by one-way ANOVA with Bonferroni as the post-test and $p<0.05$ was considered statistically significant.

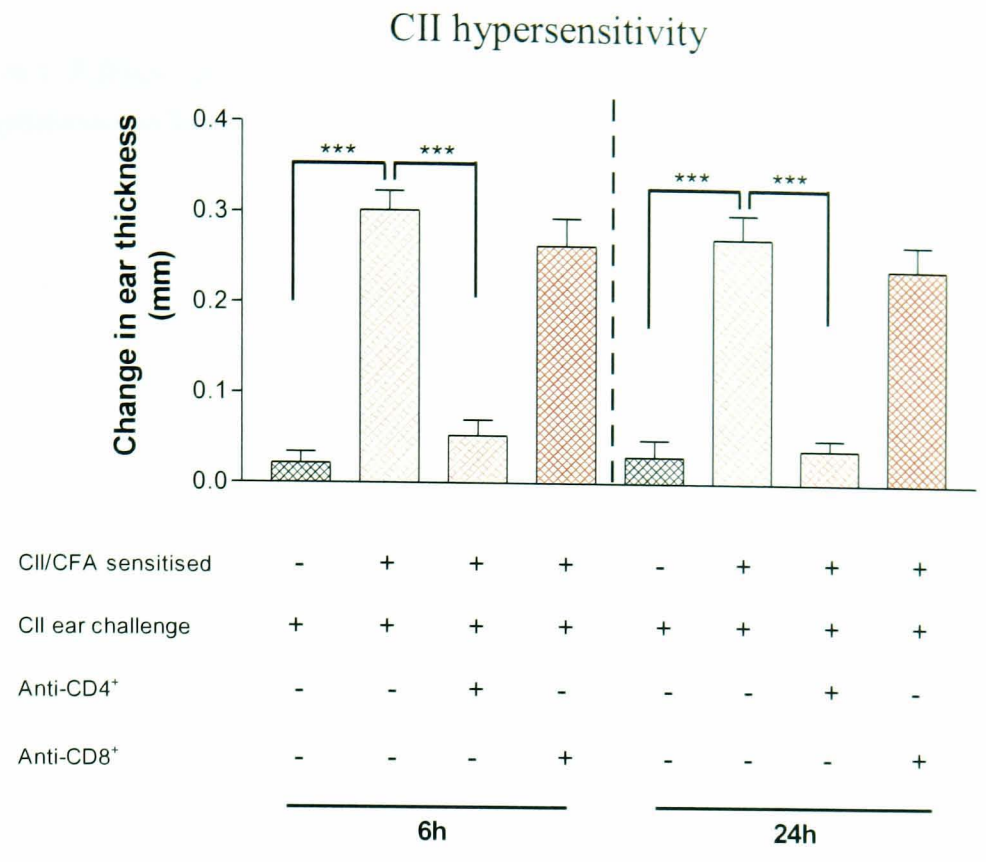
5.3.1 $CD4^+$ and $CD8^+$ T cell depletion

The role of $CD4^+$ and $CD8^+$ T cells in the CII hypersensitivity model was assessed in the same study using the depleting mAbs anti-$CD4^+$ and anti-$CD8^+$ (Figure 5.1a). Normal mice showed no statistically significant response to CII ear challenge. However, mice sensitised with CII showed a statistically significant increase in ear thickness in response to CII challenge, as compared to normal animals, from 0.022 ± 0.012 to 0.302 ± 0.022 mm at 6 hours and from 0.030 ± 0.018 to 0.272 ± 0.027 mm at 24 hours post-challenge. Anti-$CD4^+$ treatment resulted in a statistically significant inhibition in CII-induced ear swelling at 6 and 24 hours post-challenge, from 0.302 ± 0.022 to 0.053 ± 0.017 and from 0.272 ± 0.027 to 0.039 ± 0.011 mm respectively. Anti-$CD8^+$ treatment had no inhibitory effect on CII hypersensitivity either at 6 or 24 hours post-challenge.

Terminal serum samples were analysed for the presence of anti-CII Ab isotypes by ELISA (Figure 5.1b). Mice sensitised to CII showed an increase in anti-CII IgG1 production as compared to normal mice from 0.089 ± 0.018 to 0.474 ± 0.130 OD at 450nm wavelength. In the same serum samples a statistically significant increase in anti-CII IgG2a production was detected as compared to normal mice, from 0.023 ± 0.001 to 1.730 ± 0.392 OD. Anti-$CD4^+$ mAb treatment caused a decrease in anti-CII IgG1 production as compared to vehicle treated CII sensitised mice, from 0.474 ± 0.130 to 0.068 ± 0.002 OD. Furthermore, anti-$CD4^+$ mAb treatment caused a statistically significant inhibition in the production of anti-CII IgG2a as compared to vehicle treated CII sensitised mice, from 1.730 ± 0.392 to 0.026 ± 0.005 OD. Anti-$CD8^+$ treatment failed to inhibit CII specific Ab production.

The ability of these mAbs to deplete $CD4^+$ and $CD8^+$ T cells was shown in Chapter 3. In accordance with this, FACS analysis of cells from lymph nodes of treated mice in the current study also showed that $CD4^+$ and $CD8^+$ T cells had been depleted (data not shown).

a.


CII hypersensitivity
Change in ear thickness (mm)
0.4
0.3
0.2
0.1
0.0
***
***
***
***
CII/CFA sensitised - + + + - + + +
CII ear challenge + + + + + + + +
Anti-CD4+ - - + - - - + -
Anti-CD8+ - - - + - - - +
6h
24h

b.

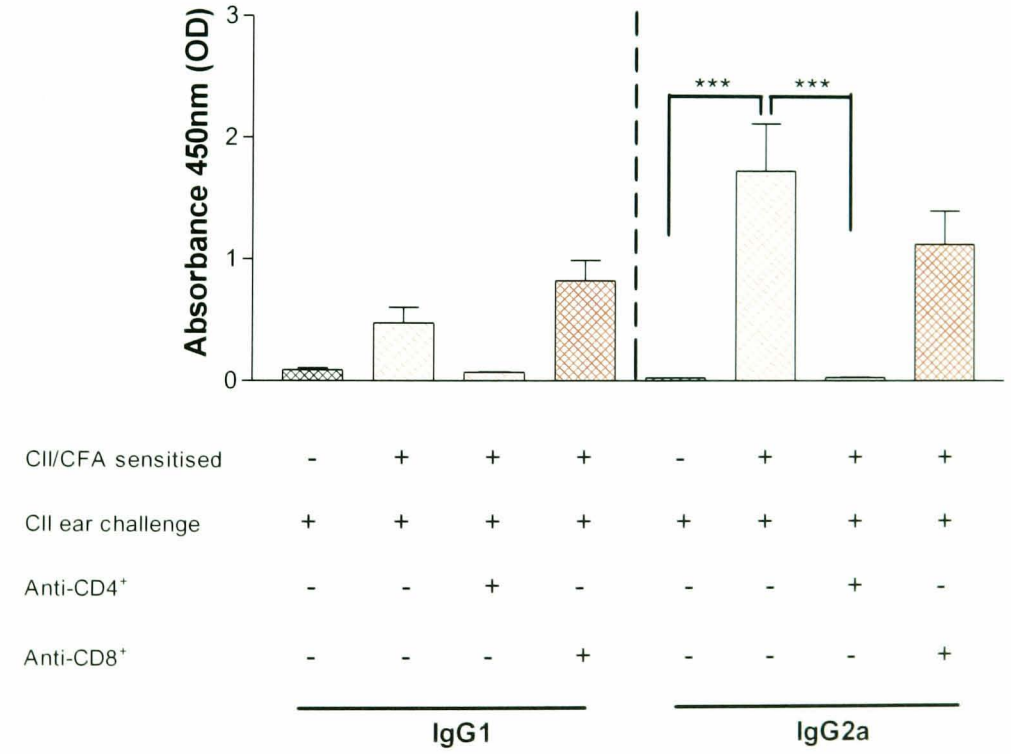

Anti-CII IgG1 and IgG2a
Absorbance 450nm (OD)
3
2
1
0
***
***
CII/CFA sensitised - + + + - + + +
CII ear challenge + + + + + + + +
Anti-CD4+ - - + - - - + -
Anti-CD8+ - - - + - - - +
IgG1
IgG2a

Figure 5.1 Effect of anti-CD4$^+$ and anti-CD8$^+$ antibody treatment on collagen II (CII) hypersensitivity and anti-CII antibody levels.

Collagen II (CII) stimulated ear swelling was assessed in normal male DBA/1 mice and in mice 14 days after sensitisation with CII in complete Freund's adjuvant. CII hypersensitivity is the change in ear swelling between challenge only and sensitised/challenged mice. Animals were challenged with 20µg CII in 25µl phosphate buffered saline (PBS) injected into the right ear. The left ear was injected with PBS only as a control. Ear swelling was measured using callipers at 6 and 24 hours post-challenge (a). At termination of the experiment (Day 15) blood samples were taken via cardiac puncture and serum collected. Anti-CII IgG1 and IgG2a (b) production was detected in serum samples by ELISA as described in Chapter 2. In this experiment some animals were dosed with anti-mouse CD4$^+$ or anti-mouse CD8$^+$ antibody at 10mg/kg s.c. on 3 consecutive days prior to sensitisation and thereafter once a week. Data presented as mean ± s.e.m, n = 5-10 per group, *** $p<0.001$ statistically significant difference from indicated control.

5.3.2 Leflunomide

To investigate if a known anti-arthritic drug has an effect on CII immunity prior to disease onset leflunomide was assessed in the CII hypersensitivity model. Leflunomide when dosed at 3mg/kg orally once a day resulted in a statistically significant inhibition in the CIA model (Chapter 3). However, when used in this model, a dose of 3mg/kg failed to inhibit CII-induced ear swelling (historical data). A further study was conducted with a dose of 10mg/kg (Figure 5.2a). In this study, normal mice showed no response to CII ear challenge. CII sensitised mice showed a statistically significant increase in ear thickness at 6 and 24 hours post-CII challenge, as compared to normal mice (from 0.044 ± 0.012 to 0.254 ± 0.008 and 0.024 ± 0.010 to 0.230 ± 0.030 mm respectively). Leflunomide caused a statistically significant inhibition in CII-induced hypersensitivity from 0.254 ± 0.008 to 0.127 ± 0.025 and 0.230 ± 0.030 to 0.089 ± 0.014 mm at 6 and 24 hours respectively. Terminal serum samples were analysed for the presence of anti-CII IgG1 and anti-CII IgG2a. CII sensitised mice showed a statistically significant increase in both anti-CII IgG1 and anti-CII IgG2a production as compared to normal mice (0.055 ± 0.001 to 0.839 ± 0.134 and 0.075 ± 0.001 to 1.940 ± 0.229 OD respectively). Leflunomide treatment resulted in a statistically significant inhibition in anti-CII IgG1 (0.839 ± 0.134 to 0.152 ± 0.041 OD) and anti-CII IgG2a (1.940 ± 0.229 to 0.279 ± 0.090 OD) production (Figure 5.2b).

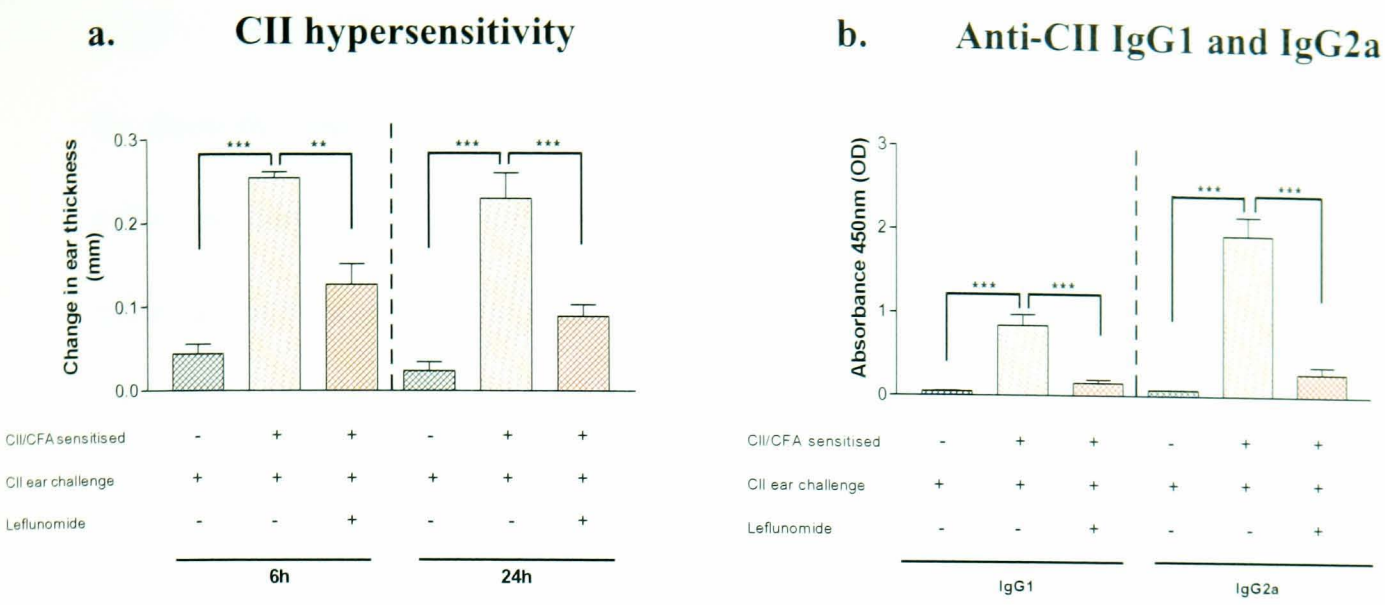


Figure 5.2 Effect of leflunomide treatment on collagen II (CII) hypersensitivity and anti-CII antibody levels.

Collagen II (CII) stimulated ear swelling was assessed in normal male DBA/1 mice and in mice 14 days after sensitisation with CII in complete Freund's adjuvant. CII hypersensitivity is the change in ear swelling between challenge only and sensitised/challenged mice. Animals were challenged with 20μg CII in 25μl phosphate buffered saline (PBS) injected into the right ear. The left ear was injected with PBS only as a control. Ear swelling was measured using callipers at 6 and 24 hours post-challenge (a). At termination of the experiment (Day 15) blood samples were taken via cardiac puncture and serum collected. Anti-CII IgG1 and IgG2a (b) production was detected in serum samples by ELISA as described in Chapter 2. In this experiment some animals were dosed with leflunomide at 10mg/kg po daily from one day prior to sensitisation. Data presented as mean ± s.e.m, n = 5-10 per group, ** $p<0.01$ and *** $p<0.001$ statistically significant difference from indicated control.

5.3.3 Anti-CD40L

To show the importance of the interaction between CD40L on T cells and CD40 on B cells in the immune response to CII, anti-CD40L was assessed in the CII hypersensitivity model (Figure 5.3a). CII sensitised mice showed a statistically significant increase in ear thickness in response to CII at 6 and 24 hours post-challenge as compared to normal mice (0.076 ± 0.024 to 0.276 ± 0.007 and 0.044 ± 0.013 to 0.214 ± 0.012 mm respectively). Sensitised mice treated with anti-CD40L showed a statistically significant inhibition in this CII stimulated hypersensitivity at 6 hours (0.276 ± 0.007 to 0.053 ± 0.005 mm) and 24 hours (0.214 ± 0.012 to 0.045 ± 0.008 mm) post-challenge. Anti-CII Ab isotypes were analysed from terminal serum samples. CII sensitised mice showed a statistically significant increase in both anti-CII IgG1 and anti-CII IgG2a production as compared to normal mice (0.013 ± 0.0003 to 0.441 ± 0.053 and 0.020 ± 0.001 to 0.938 ± 0.151 OD respectively). Treatment with anti-CD40L resulted in a statistically significant abrogation of anti-CII IgG1 and anti-CII IgG2a production from 0.441 ± 0.053 to 0.014 ± 0.002 and from 0.938 ± 0.151 to 0.022 ± 0.001 OD respectively (Figure 5.3b).

5.3.4 Anti-LFA-1

To identify if the interaction between LFA-1 on T cells and ICAM on APCs is required in the immune response to CII in pre-arthritic mice, anti-LFA-1 was assessed in the hypersensitivity model (Figure 5.4a). Normal mice showed no response to CII ear challenge. However, CII sensitised animals, challenged with CII, showed a statistically significant increase in ear thickness as compared to normal mice from 0.076 ± 0.024 to 0.276 ± 0.007 and from 0.044 ± 0.013 to 0.214 ± 0.012 mm at 6 and 24 hours post-challenge respectively. Anti-LFA-1 treatment caused a statistically

significant reduction in this ear swelling from 0.276 ± 0.007 to 0.068 ± 0.012 at 6 hours and from 0.214 ± 0.012 to 0.061 ± 0.016 mm at 24 hours. Serum samples from sensitised mice showed a statistically significant increase in anti-CII IgG1 and anti-CII IgG2a production, as compared to normal mice, from 0.013 ± 0.0003 to 0.441 ± 0.053 and from 0.020 ± 0.001 to 0.938 ± 0.151 OD respectively. Anti-LFA-1 treatment caused a statistically significant inhibition in anti-CII IgG1 production from 0.441 ± 0.053 to 0.039 ± 0.016 OD and anti-CII IgG2a production from 0.938 ± 0.151 to 0.184 ± 0.075 OD (Figure 5.4b).

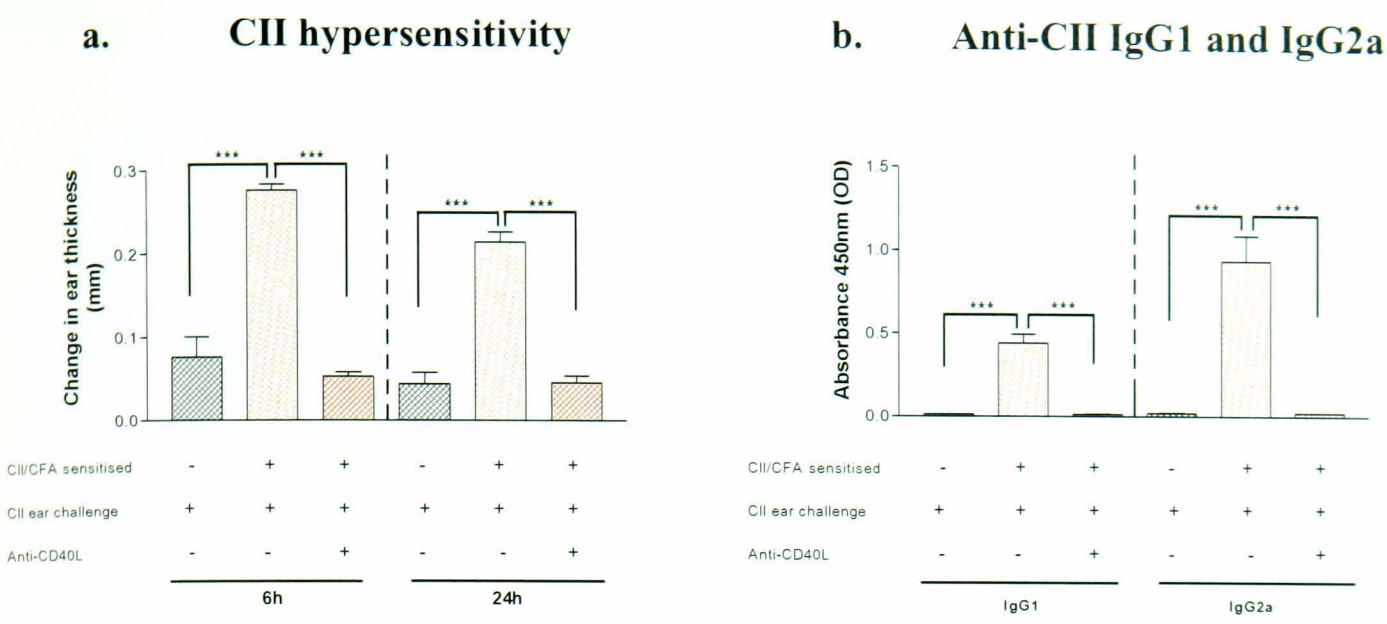


Figure 5.3 Effect of anti-CD40L antibody treatment on collagen II (CII) hypersensitivity and anti-CII antibody levels.

Collagen II (CII) stimulated ear swelling was assessed in normal male DBA/1 mice and in mice 14 days after sensitisation with CII in complete Freund's adjuvant. CII hypersensitivity is the change in ear swelling between challenge only and sensitised/challenged mice. Animals were challenged with 20µg CII in 25µl phosphate buffered saline (PBS) injected into the right ear. The left ear was injected with PBS only as a control. Ear swelling was measured using callipers at 6 and 24 hours post-challenge (a). At termination of the experiment (Day 15) blood samples were taken via cardiac puncture and serum collected. Anti-CII IgG1 and IgG2a (b) production was detected in serum samples by ELISA as described in Chapter 2. In this experiment some animals were dosed with anti-CD40L antibody at 30mg/kg s.c. once a week from one day prior to sensitisation. Data presented as mean ± s.e.m, n = 5-10 per group, *** $p<0.001$ statistically significant difference from indicated control.

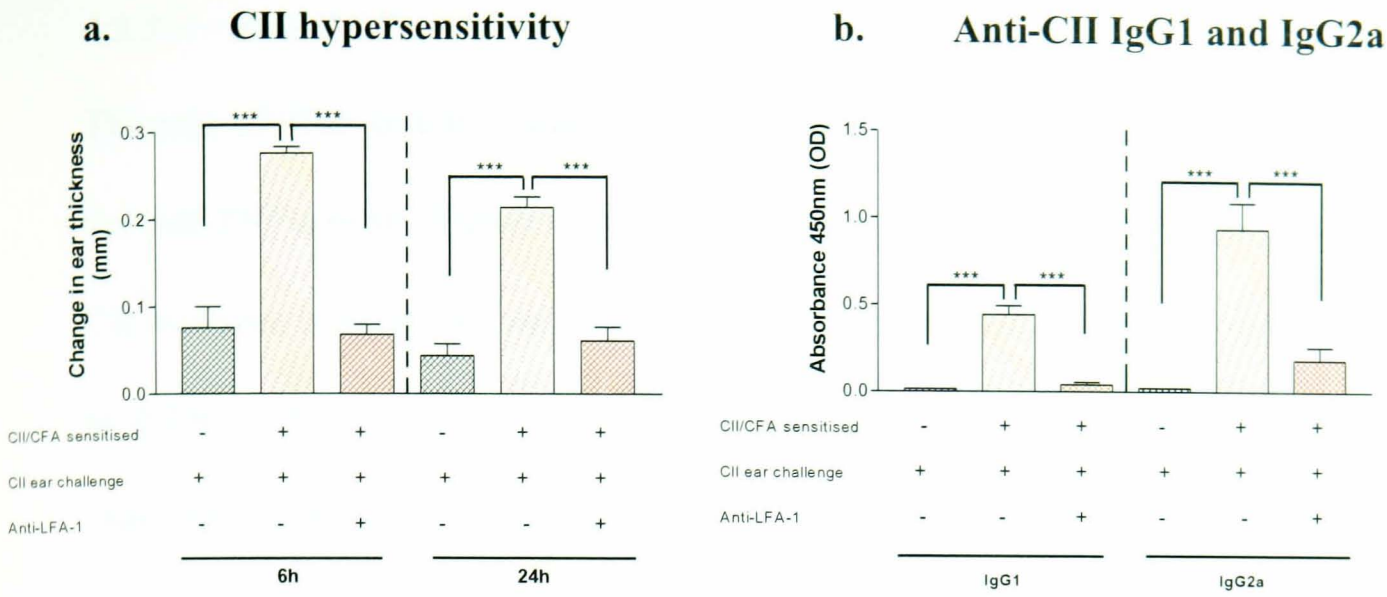


Figure 5.4 Effect of anti-LFA-1 antibody treatment on collagen II (CII) hypersensitivity and anti-CII antibody levels.

Collagen II (CII) stimulated ear swelling was assessed in normal male DBA/1 mice and in mice 14 days after sensitisation with CII in complete Freund's adjuvant. CII hypersensitivity is the change in ear swelling between challenge only and sensitised/challenged mice. Animals were challenged with 20μg CII in 25μl phosphate buffered saline (PBS) injected into the right ear. The left ear was injected with PBS only as a control. Ear swelling was measured using callipers at 6 and 24 hours post-challenge (a). At termination of the experiment (Day 15) blood samples were taken via cardiac puncture and serum collected. Anti-CII IgG1 and IgG2a (b) production was detected in serum samples by ELISA as described in Chapter 2. In this experiment some animals were dosed with anti-LFA-1 antibody at 30mg/kg s.c. once a week from one day prior to sensitisation. Data presented as mean ± s.e.m, n = 5-10 per group, *** $p<0.001$ statistically significant difference from indicated control.

5.3.5 Anti-TNFα

The role of TNF in CII immunity was assessed in the hypersensitivity model using the anti-TNFα mAb (Figure 5.5a). Statistically significant ear swelling in response to CII was seen in sensitised mice as compared to normal mice at 6 hours (0.014 ± 0.007 to 0.236 ± 0.050 mm) and 24 hours (0.004 ± 0.010 to 0.156 ± 0.050 mm) post-challenge. Anti-TNFα treatment failed to inhibit ear swelling at 6 or 24 hours. Analysis of anti-CII IgG1 and anti-CII IgG2a in terminal serum samples showed that sensitised mice produced a statistically significant increase in both Ab isotypes as compared to normal mice (0.011 ± 0.001 to 0.315 ± 0.039 and 0.024 ± 0.003 to 0.909 ± 0.154 OD respectively). Anti-TNFα treatment caused a statistically significant attenuation in this Ab response from 0.315 ± 0.039 to 0.165 ± 0.022 OD for anti-CII IgG1 and from 0.909 ± 0.154 to 0.411 ± 0.060 OD for anti-CII IgG2a (Figure 5.5b).

5.3.6 Anti-IL-1β

The role of IL-1β in the CII hypersensitivity model was assessed using the anti-IL-1β mAb (Figure 5.6a). A statistically significant increase in ear swelling was seen in response to CII in sensitised mice as compared to normal mice at 6 and 24 hours post-challenge (0.076 ± 0.024 to 0.276 ± 0.007 and 0.044 ± 0.013 to 0.214 ± 0.012 mm respectively). Anti-IL-1β treatment had no effect on ear swelling at 6 hours post-challenge. However, at 24 hours there was a statistically significant reduction in CII-induced hypersensitivity from 0.214 ± 0.012 to 0.153 ± 0.017 mm. Anti-CII IgG1 and anti-CII IgG2a production was analysed in terminal serum samples and sensitised mice showed a statistically significant increase in both Ab isotypes as compared to normal mice (0.011 ± 0.001 to 0.315 ± 0.039 and 0.024 ± 0.003 to 0.909 ± 0.154 OD

respectively). Treatment with anti-IL-1β had no effect on anti-CII IgG1 or anti-CII IgG2a production (Figure 5.6b).

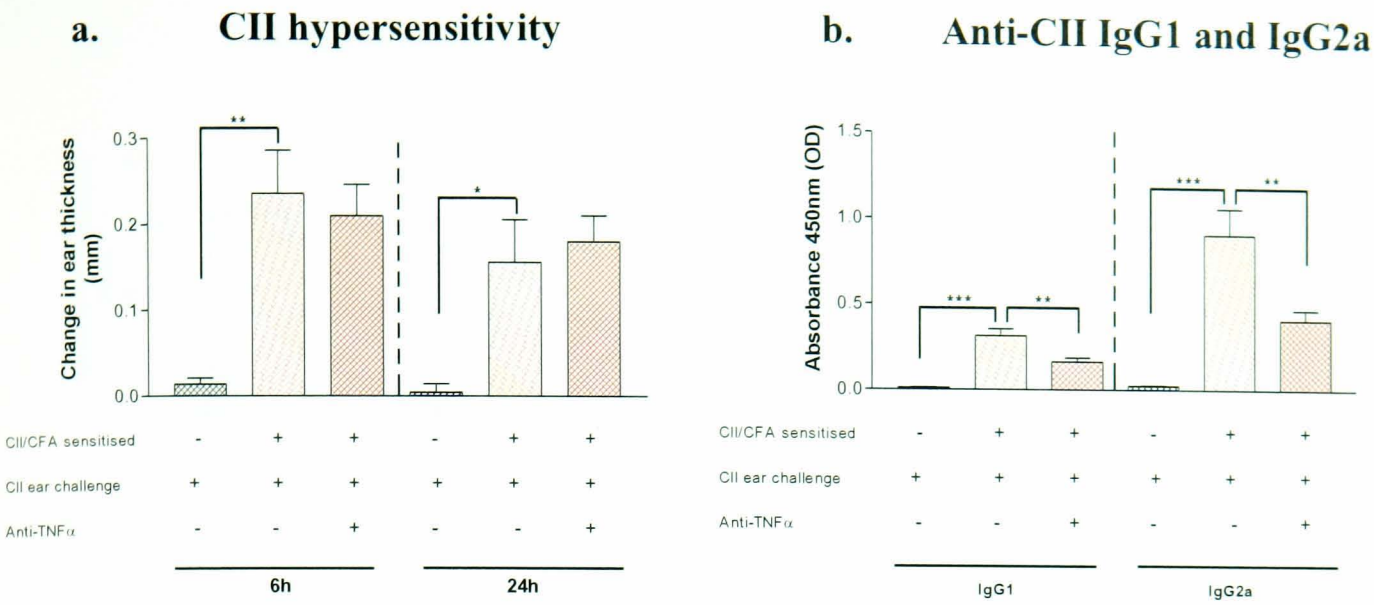


Figure 5.5 Effect of anti-TNFα antibody treatment on collagen II (CII) hypersensitivity and anti-CII antibody levels.

Collagen II (CII) stimulated ear swelling was assessed in normal male DBA/1 mice and in mice 14 days after sensitisation with CII in complete Freund's adjuvant. CII hypersensitivity is the change in ear swelling between challenge only and sensitised/challenged mice. Animals were challenged with 20μg CII in 25μl phosphate buffered saline (PBS) injected into the right ear. The left ear was injected with PBS only as a control. Ear swelling was measured using callipers at 6 and 24 hours post-challenge (a). At termination of the experiment (Day 15) blood samples were taken via cardiac puncture and serum collected. Anti-CII IgG1 and IgG2a (b) production was detected in serum samples by ELISA as described in Chapter 2. In this experiment some animals were dosed with anti-mouse TNFα antibody at 100mg/kg s.c. twice a week from one day prior to sensitisation. Data presented as mean ± s.e.m, n = 5-10 per group, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ statistically significant difference from indicated control.

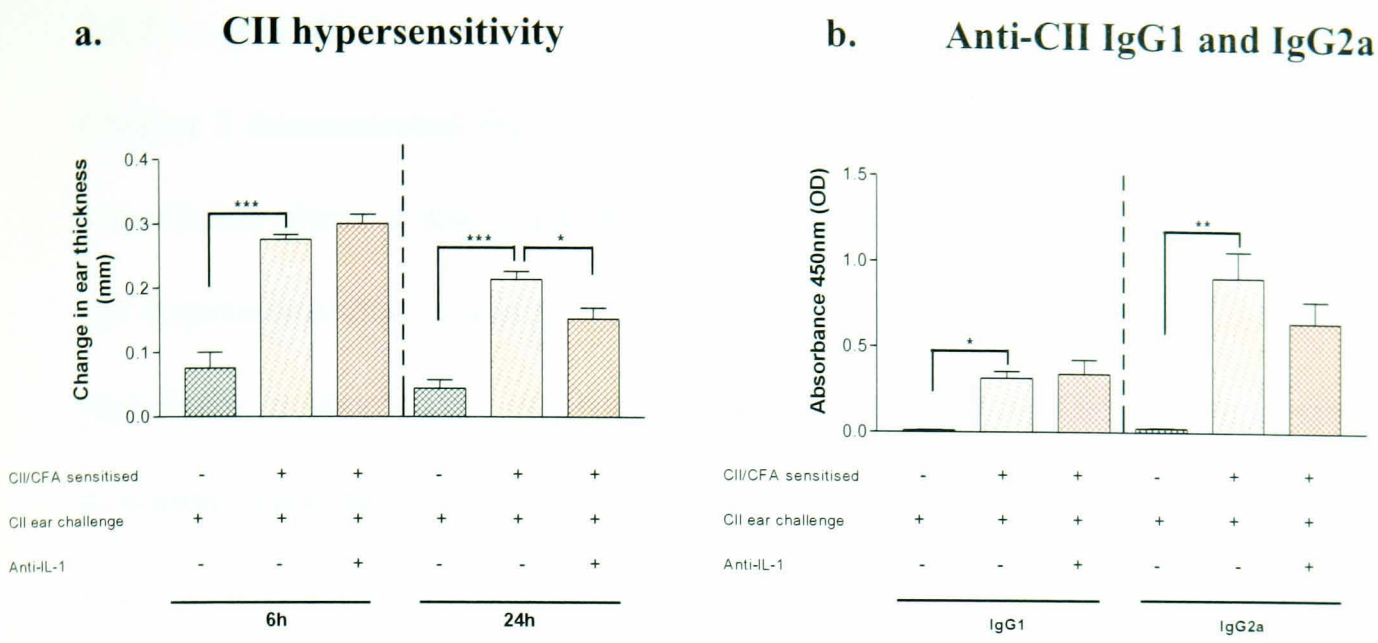


Figure 5.6 Effect of anti-IL-1β antibody treatment on collagen II (CII) hypersensitivity and anti-CII antibody levels.

Collagen II (CII) stimulated ear swelling was assessed in normal male DBA/1 mice and in mice 14 days after sensitisation with CII in complete Freund's adjuvant. CII hypersensitivity is the change in ear swelling between challenge only and sensitised/challenged mice. Animals were challenged with 20μg CII in 25μl phosphate buffered saline (PBS) injected into the right ear. The left ear was injected with PBS only as a control. Ear swelling was measured using callipers at 6 and 24 hours post-challenge (a). At termination of the experiment (Day 15) blood samples were taken via cardiac puncture and serum collected. Anti-CII IgG1 and IgG2a (b) production was detected in serum samples by ELISA as described in Chapter 2. In this experiment some animals were dosed with anti-mouse IL-1β antibody at 10mg/kg s.c. once a week from one day prior to sensitisation. Data presented as mean ± s.e.m, n = 5-10 per group, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ statistically significant difference from indicated control.

5.3.7 Anti-IL-6

Chapter 3 demonstrated the role of IL-6 in CIA. To establish if CII immunity in the pre-arthritic phase is also dependent on this cytokine the mAb anti-IL-6 was tested in the hypersensitivity model (Figure 5.7a). In this study there was a statistically significant increase in ear swelling in response to CII in sensitised mice as compared to normal mice, from 0.076 ± 0.024 to 0.276 ± 0.007 mm at 6 hours and from 0.044 ± 0.013 to 0.214 ± 0.012 mm at 24 hours post-challenge. Anti-IL-6 treatment had no effect on ear swelling at 6 hours post-challenge but did cause a statistically significant attenuation in ear swelling at 24 hours from 0.214 ± 0.012 to 0.152 ± 0.012 mm. Analysis of terminal serum samples revealed that there was a statistically significant increase in anti-CII IgG1 and anti-CII IgG2a production in sensitised mice as compared to normal mice (0.060 ± 0.002 to 0.6568 ± 0.070 and 0.068 ± 0.002 to 1.178 ± 0.183 OD respectively). Treatment with anti-IL-6 resulted in a statistically significant inhibition of this response from 0.6568 ± 0.070 to 0.263 ± 0.043 OD for anti-CII IgG1 and from 1.178 ± 0.183 to 0.574 ± 0.085 OD for anti-CII IgG2a production (Figure 5.7b).

5.3.8 Anti-IL-17

IL-17 represents a novel therapeutic target and it was shown in Chapter 3 that a mAb directed against it inhibits CIA. To establish the role of this cytokine in CII immunity and to identify if the anti-arthritic effects of anti-IL-17 can be detected in pre-arthritic readouts of CII immunity, this mAb was assessed in the hypersensitivity model (Figure 5.8a). A statistically significant increase in ear swelling in response to CII challenge was seen in sensitised mice as compared to normal mice at 6 hours (0.076 ± 0.024 to 0.276 ± 0.007 mm) and 24 hours (0.044 ± 0.013 to 0.214 ± 0.012 mm)

post-challenge. Anti-IL-17 treatment did not inhibit CII-induced ear swelling at 6 hours post-challenge but did cause a statistically significant inhibition in ear swelling at 24 hours post-challenge from 0.214 ± 0.012 to 0.150 ± 0.012 mm. Terminal serum samples were analysed for the presence of anti-CII IgG1 and anti-CII IgG2a production and sensitised mice showed a statistically significant increase in both Ab isotypes as compared to normal mice (0.060 ± 0.002 to 0.657 ± 0.070 and 0.068 ± 0.002 to 1.178 ± 0.183 OD respectively). Anti-IL-17 treatment had no effect on anti-CII IgG1 or anti-CII IgG2a production (Figure 5.8b).

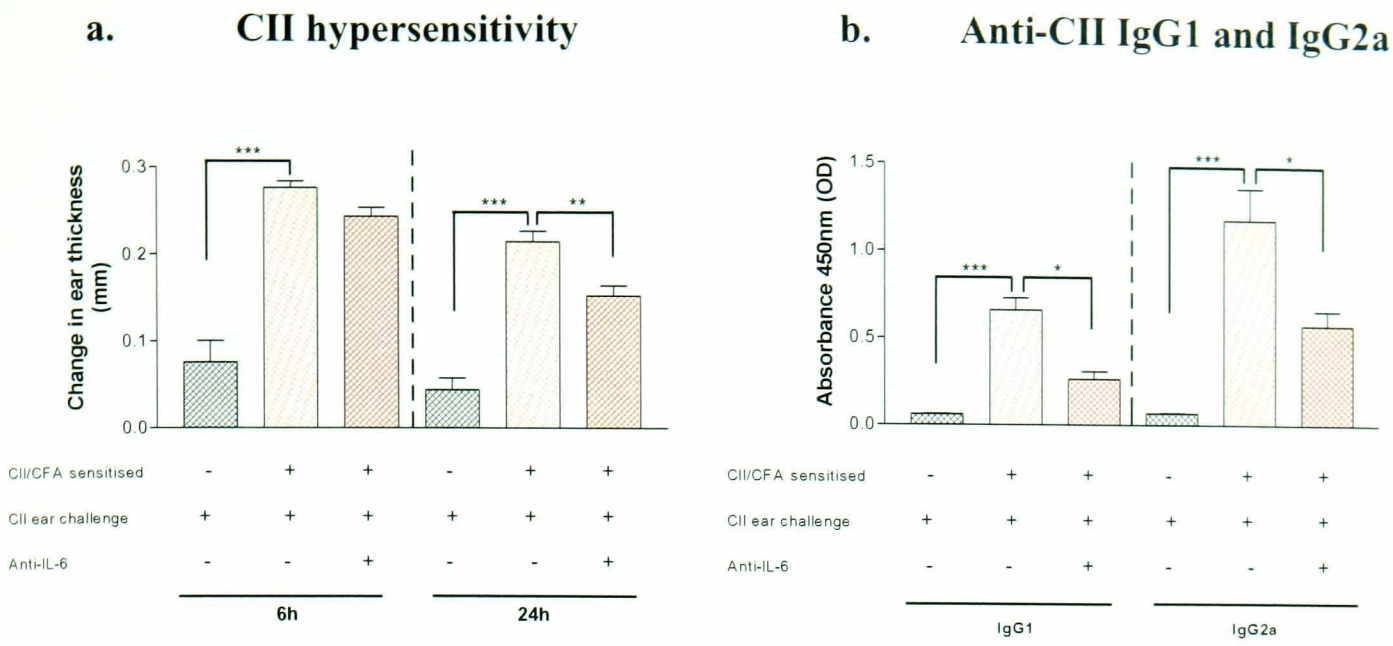


Figure 5.7 Effect of anti-IL-6 antibody treatment on collagen II (CII) hypersensitivity and anti-CII antibody levels.

Collagen II (CII) stimulated ear swelling was assessed in normal male DBA/1 mice and in mice 14 days after sensitisation with CII in complete Freund's adjuvant. CII hypersensitivity is the change in ear swelling between challenge only and sensitised/challenged mice. Animals were challenged with 20μg CII in 25μl phosphate buffered saline (PBS) injected into the right ear. The left ear was injected with PBS only as a control. Ear swelling was measured using callipers at 6 and 24 hours post-challenge (a). At termination of the experiment (Day 15) blood samples were taken via cardiac puncture and serum collected. Anti-CII IgG1 and IgG2a (b) production was detected in serum samples by ELISA as described in Chapter 2. In this experiment some animals were dosed with anti-mouse IL-6 antibody at 10mg/kg s.c. once a week from one day prior to sensitisation. Data presented as mean ± s.e.m, n = 5-10 per group, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ statistically significant difference from indicated control.

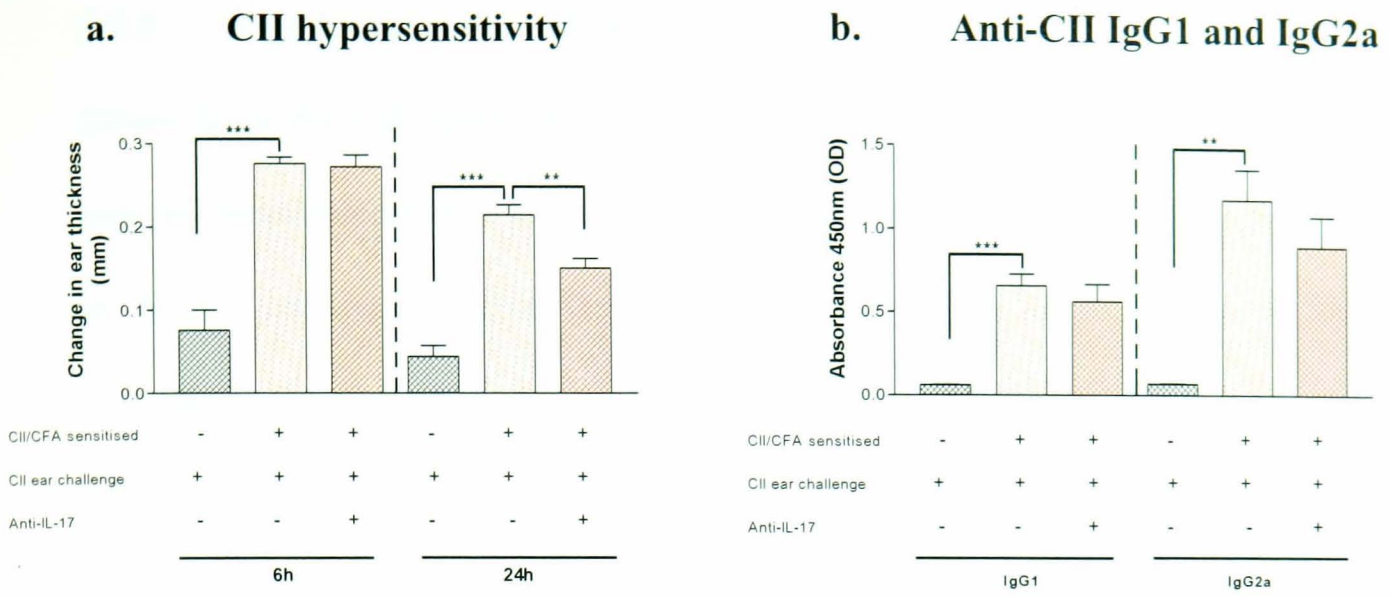


Figure 5.8 Effect of anti-IL-17 antibody treatment on collagen II (CII) hypersensitivity and anti-CII antibody levels.

Collagen II (CII) stimulated ear swelling was assessed in normal male DBA/1 mice and in mice 14 days after sensitisation with CII in complete Freund's adjuvant. CII hypersensitivity is the change in ear swelling between challenge only and sensitised/challenged mice. Animals were challenged with 20μg CII in 25μl phosphate buffered saline (PBS) injected into the right ear. The left ear was injected with PBS only as a control. Ear swelling was measured using callipers at 6 and 24 hours post-challenge (a). At termination of the experiment (Day 15) blood samples were taken via cardiac puncture and serum collected. Anti-CII IgG1 and IgG2a (b) production was detected in serum samples by ELISA as described in Chapter 2. In this experiment some animals were dosed with anti-mouse IL-17 antibody at 10mg/kg s.c. once a week from one day prior to sensitisation. Data presented as mean ± s.e.m, n = 5-10 per group, ** $p<0.01$ and *** $p<0.001$ statistically significant difference from indicated control.

5.3.9 SAA levels in the CII-induced hypersensitivity model

Serum from the CII hypersensitivity studies were analysed for levels of SAA. This analysis demonstrated that a CII ear challenge in sensitised mice produced an increase in SAA production above that of normal mice (51.45 ± 26.78 to 733.30 ± 370.40μg/mL). However, this response was highly variable between mice and did not result in a statistically significant difference. Thus, SAA levels on Day 15 in the CII-induced hypersensitivity model could not be utilised as a robust readout and drug effects were therefore not assessed.

5.4 Discussion

Table 5.1 summarises drug activity in the CIA model, CII hypersensitivity model and anti-CII Ab assay.

Drug	CIA	Hypersensitivity		Anti-CII antibodies
		6h	24h	
Anti-CD4$^+$	+ **(100)**	+	+	+
Anti-CD8$^+$	- **(19)**	-	-	-
Leflunomide	+ **(68)**	+	+	+
Anti-CD40L	+ **(81)**	+	+	+
Anti-LFA-1	- **(38)**	+	+	+
Anti-TNFα	+ **(82)**	-	-	+
Anti-IL-1β	+ **(100)**	-	+	-
Anti-IL-6	+ **(87)**	-	+	+
Anti-IL-17	+ **(75)**	-	+	-

Table 5.1 Summary of drug effects on CIA, CII-induced hypersensitivity and anti-CII antibody production.

Note: *+ represents a statistically significant inhibition in CIA, CII-induced hypersensitivity and anti-CII IgG1 or IgG2a as compared to control.*

- represents no significant change from control.

The numbers in brackets represent the percent reduction in the AUC of the clinical score as compared to controls.

Refer to Chapter 3 for CIA data.

In Chapter 3 it was demonstrated that CIA is driven by CD4$^+$ T cells and that CD8$^+$ T cells are not important in the development of arthritis. The role of these T cell subsets in CII immunity prior to disease onset was investigated in this chapter. Anti-CD4$^+$

treatment caused a statistically significant inhibition in CII hypersensitivity and anti-CII Ab production, illustrating that CII immunity in the pre-arthritic phase, like CIA, is $CD4^+$ T cell dependent. Given the role of $CD4^+$ T cells in adaptive immunity, it is not surprising that their depletion resulted in such profound effects. Administration of the anti-$CD8^+$ mAb had no effect on CII hypersensitivity or anti-CII Ab production demonstrating that $CD8^+$ T cells do not play a role in CII immunity. These data are supportive of a lack of anti-arthritic activity seen in CIA. It is evident from these readouts that $CD4^+$ T cells are essential in the development of an immune response to CII and hence CIA.

To further investigate the underlying immune processes involved in CII immunity, the role of CD40L and LFA-1 were investigated. Anti-CD40L treatment in the hypersensitivity model caused a statistically significant inhibition in ear swelling and anti-CII Ab production. This demonstrates that the interaction between T and B cells is important in the immune response to CII in this model as well as in CIA. The data reported here demonstrates the role of CD40L in the production of anti-CII IgG1 and anti-CII IgG2a. In support of this, Durie *et al.* (1993) reported a reduction in anti-CII IgG1 levels after CD40L blockade in CIA. However, Durie *et al* did not identify this inhibitory effect at such an early time point.

The interaction between T cells and APCs in these early readouts of CII immunity was assessed using the anti-LFA-1 mAb. Anti-LFA-1 treatment caused a statistically significant inhibition in ear swelling and anti-CII Ab production in this model. These results were surprising as it was anticipated that anti-LFA-1, having failed to inhibit CIA, would also fail to suppress CII immunity in the pre-arthritic phase. It therefore

appears that LFA-1 is an important integrin in the early immune response to CII but as disease progresses its function is lost.

Anti-$CD4^+$, anti-CD40L and anti-LFA-1 treatment all caused a suppression in the humoral response to CII. This immunosuppressive effect was associated with attenuation in the immediate type hypersensitivity response at 6 hours post-challenge and is therefore supportive of this readout being Ab mediated. In addition, the depletion of $CD4^+$ T cells and the blockade of APC - T cell contact were associated with an inhibition in the 24 hour hypersensitivity response, suggesting this readout is T cell mediated. Furthermore, anti-CD40L inhibited the 24 hour readout which suggests that CD40L may also be involved in T cell activation as well as Ab production.

In order to validate the CII hypersensitivity model, leflunomide, a clinically used DMARD, was assessed. In Chapter 3 it was demonstrated that leflunomide dosed at 3mg/kg inhibited CIA, however this dose had no effect in the CII hypersensitivity model. Increasing the dose of leflunomide to 10mg/kg in the current study caused a statistically significant inhibition in CII-induced hypersensitivity and anti-CII Ab production. This suggests that the hypersensitivity model is not as sensitive as the CIA model and that the immune response to CII in the pre-arthritic phase is harder to inhibit. The inhibitory effect of leflunomide in this model indicates that lymphocyte proliferation is important in the early immune response to CII and has demonstrated that a clinically relevant drug is active in this short-term model.

Anti-TNFα, anti-IL-1β and anti-IL-6 mAbs were used in this chapter to further validate this short-term model of CII immunity. Anti-TNFα treatment is effective in

the CIA model (Chapter 3), however in the current model it failed to inhibit CII-induced ear swelling at 6 or 24 hours post-challenge. The lack of effect at 24 hours suggests that anti-TNFα did not have an effect on cellular immunity in this model. However, it did cause a statistically significant inhibition in anti-CII Ab production, something which has not been reported previously. This decrease in anti-CII Ab production did not, however, translate into a reduction in the 6 hour hypersensitivity response. This lack of effect at 6 hours may be explained as anti-TNFα did not completely suppress anti-CII Ab production thus there is enough circulating IgG to elicit an immediate type hypersensitivity response to CII. In addition, the amount of CII used to induce ear swelling may be too high making it more difficult to suppress. This supports the observation made above that the model is not as sensitive as CIA.

TNFα is reported to be a pro-inflammatory cytokine and Williams *et al.* (1992) had previously shown that anti-TNFα treatment has no effect on anti-CII IgG production in CIA. Thus, the identification of an immunomodulatory effect of anti-TNFα was unexpected. In addition, the study by Williams *et al* assessed anti-CII IgG production in the arthritic phase which suggests this pre-arthritic model may give an insight into drug mechanism of action that could be lost later on. The reduction in anti-CII Ab production detected in the current study may, at least in part, contribute to the anti-arthritic activity of anti-TNFα in the CIA model and represents a novel mechanism of action.

Administration of anti-IL-1β failed to inhibit CII-induced hypersensitivity 6 hours post-challenge and also failed to suppress anti-CII Ab production, suggesting it does not affect humoral immunity. However, it did result in a statistically significant inhibition in CII-induced hypersensitivity 24 hours post-challenge. This suggests that

anti-IL-1β may be exerting some of its anti-arthritic effect in the CIA model by inhibiting cellular immunity to CII.

Anti-IL-6 treatment caused a statistically significant inhibition in CII-induced hypersensitivity at 24 hours post-challenge and anti-CII Ab production, therefore demonstrating an essential role for IL-6 in CII immunity. These data strongly suggest that the anti-arthritic activity of anti-IL-6 in the CIA model is mediated through its immunomodulatory effects on both cellular and humoral immunity. These results were not surprising given the reported role of IL-6 on B cell differentiation, Ab production and T cell proliferation (Splawshi *et al.*, 1990, Croft and Swain. 1991 and Pankewycz *et al.*, 1990 respectively). However, anti-IL-6 treatment did not inhibit CII-induced hypersensitivity at 6 hours post-challenge and it did not completely suppress anti-CII Ab production. This is similar to the results seen with anti-TNFα and the same arguments regarding the model can be applied. In addition, anti-IL-6 and anti-IL-1β treatment failed to completely attenuate hypersensitivity at 24 hours post-challenge. This may suggest that these drugs only have a partial effect on cellular immunity or that this time point, like the 6 hour one, is much harder to inhibit. The data from the anti-cytokine drugs has demonstrated that CII immunity in the pre-arthritic phase is dependent at least in part on TNFα, IL-1β and IL-6.

The role of IL-17 in CII immunity was assessed using the anti-IL-17 mAb. This Ab had no effect on the production of anti-CII IgG1 or anti-CII IgG2a and did not prevent CII-induced hypersensitivity at 6 hours post-challenge. Anti-IL-17 treatment did cause a statistically significant inhibition in hypersensitivity when measured at 24 hours post-challenge. This early effect of blocking IL-17 on CII immunity has not been reported before. These data suggest that the anti-arthritic activity of anti-IL-17

in CIA may be due in part to its effect on the cellular immune response to CII. It is evident that IL-17, like the other cytokines investigated, has a role to play in CII immunity. Moreover, it has been demonstrated that these early readouts of CII immunity have the potential to detect the activity of novel therapies.

This chapter has demonstrated that CII-induced hypersensitivity and anti-CII Ab production in pre-arthritic mice, like CIA, are dependent on $CD4^+$ T cells and T cell-B cell contact and proliferation. However, unlike CIA, LFA-1 seems to play an essential role in these short-term readouts and demonstrates the importance of T cell-APC interactions in CII immunity in the pre-arthritic phase. These short-term readouts have also been validated using leflunomide and mAbs that target TNFα, IL-1β and IL-6 suggesting that they are clinically relevant.

The data presented here (Table 5.1) has shown that, in general, drugs which had anti-arthritic activity in the CIA model also had inhibitory effects on CII-induced hypersensitivity and/or anti-CII Ab production. Furthermore, anti-$CD8^+$ treatment, which had no effect on CIA, also had no effect on these readouts. Thus, it appears these early readouts of CII immunity are capable of predicting anti-arthritic drug effects in CIA and have the potential to be utilised as part of a screening cascade in drug discovery. However, there was one notable exception, anti-LFA-1, which failed to cause a statistically significant inhibition in CIA, but did have a profound effect in reducing both CII-induced hypersensitivity and anti-CII Ab production. If these readouts were incorporated into a screening cascade, key findings would be followed up in the CIA model. In respect of anti-LFA-1 it would become clear that the hypersensitivity and anti-CII Ab data represented false positive results in terms of

their predictive value. From a drug discovery point of view, this is much better than having a false negative result, where active drugs may be overlooked.

It has also become apparent that these readouts should be used in combination when assessing novel therapies as anti-TNFα only inhibited anti-CII Ab production whereas anti-IL-1β and anti-IL-17 only inhibited CII-induced hypersensitivity at 24 hours. If used in isolation, the potential anti-arthritic properties of some drugs may not be detected.

The differing effect between these drugs has, however, demonstrated that these readouts of CII immunity are capable of discriminating between drugs based on mechanism of action. In respect to anti-TNFα, it appears it may be exerting some of its anti-arthritic effect in the CIA model by inhibiting the humoral immune response to CII. Anti-IL-1β and anti-IL-17, on the other hand, may be exerting some of their anti-arthritic effect by inhibiting the cellular immune response to CII. Unfortunately, the assessment of anti-CII IgG1 and anti-CII IgG2a did not infer any differential drug effects on Th2 and Th1 cell responses respectively and drugs that had an effect on humoral immunity suppressed the production of both isotypes. This suggests that drugs may be either inhibiting both Th1 and Th2 cell activity or having a direct effect on antibody production.

Overall, the CII-induced hypersensitivity model and the anti-CII Ab assay appear to represent a promising early screen for novel anti-arthritic drugs. In addition, there appears to be less variability in these readouts and a reduced number of animals were used as compared to the CIA model to obtain robust and reproducible data. This, in conjunction with animals not being rendered arthritic, has clear welfare advantages as compared to CIA.

It has been indicated in this chapter that a number of drugs studied have an effect on the cellular immune response to CII. In order to confirm these effects drugs were assessed in the following chapter in the *ex vivo* CII stimulated $CD4^+$ T cell thymidine incorporation assay. These drugs were also used to characterise this assay and to determine if it is predictive of the CIA model.

Chapter six

Drug effects on CII stimulated $CD4^+$ T cell proliferation

6.1 Introduction

It has been demonstrated in pre-arthritic mice that humoral and cellular readouts of CII immunity, such as anti-CII Ab production and CII-induced hypersensitivity appear, on the whole, to be predictive of anti-arthritic drug effects in CIA (Chapter 5). In addition, data from the CII-induced hypersensitivity model suggested that a number of the drugs under investigation in this thesis may have an effect on the cellular immune response to CII. In this chapter, drugs were assessed in the *ex vivo* CII stimulated $CD4^+$ T cell thymidine incorporation assay to confirm their effects on cellular immunity to CII and to determine if this short-term readout is predictive of anti-arthritic drug activity. Anti-CD40L and anti-LFA-1 mAbs were used to investigate the underlying immune processes involved in CII stimulated $CD4^+$ T cell thymidine incorporation. Leflunomide, anti-TNFα, anti-IL-1β and anti-IL-6 were used for validation. Once characterised, the role of IL-17 in this short-term cellular readout of CII immunity was investigated using the anti-IL-17 mAb. Moreover, the use of anti-IL-17 will identify if this assay can be used to assess the activity of novel therapeutics.

Drugs were administered at the same dose and frequency as outlined in Chapter 5. In addition to assessing *in vivo* drug effects on *ex vivo* CII stimulated $CD4^+$ T cell thymidine incorporation, drugs were also added *in vitro* (where possible) to cultures of cells from CII sensitised animals. Assessment of drugs both *in vivo* and *in vitro* in this assay may help to identify whether drugs are having an effect on the generation of $CD4^+$ T cells or whether they are capable of influencing $CD4^+$ T cells that are already activated. Furthermore, this cellular readout of CII immunity may help to discriminate drugs based on their mechanisms of action.

6.2 Aim

The aim of this chapter was to characterise and validate the CII stimulated $CD4^+$ T cell thymidine incorporation assay with the drugs utilised in Chapter 3 and to determine if this readout can predict and discriminate anti-arthritic drug effects.

6.3 Results

In the following set of experiments the incorporation of tritiated thymidine into $CD4^+$ T cells measured as counts per minute (cpm) was used as a surrogate marker of proliferation. A statistically significant increase in cpm between non-stimulated and CII stimulated cells isolated from vehicle treated CII/CFA sensitised mice was considered to represent a proliferative response. Drugs were assessed either *in vivo* in CII/CFA sensitised mice or *in vitro* on cells from vehicle treated CII/CFA sensitised mice, for their ability to reduce this response. Data is presented as mean cpm per group. Statistical analysis of the data was conducted by one-way ANOVA with Bonferroni as the post-test and $p<0.05$ was considered statistically significant.

6.3.1 Leflunomide

In order to validate this assay, a known inhibitor of lymphocyte proliferation, leflunomide (Chong *et al.*, 1993), was assessed on CII stimulated $CD4^+$ T cell thymidine incorporation. Leflunomide has already been shown to inhibit CIA when administered at 3mg/kg (Chapter 3). In the current study leflunomide was dosed at 3 and 10mg/kg once a day (Figure 6.1). Cells from normal mice cultured in the absence of CII (non-stimulated) gave counts of 9871 ± 656 cpm and the same cells did not respond to *in vitro* CII stimulation as indicated by a lack of increased thymidine incorporation (9024 ± 897 cpm). Cells cultured from CFA sensitised mice showed an increase in thymidine incorporation to 21135 ± 918 cpm. However, no further increase was seen when these cells were cultured with CII (20500 ± 1157 cpm). Non-stimulated cells isolated from CII/CFA sensitised mice showed similar counts to cells from CFA sensitised mice (20914 ± 1078 cpm), however, when these cells were stimulated with CII *in vitro* there was a statistically significant increase in thymidine

incorporation to 58666 ± 2530 cpm. This CII specific response was inhibited in a dose dependent manner when CII/CFA sensitised mice were treated *in vivo* with leflunomide at 3 and 10mg/kg, reducing counts to 34192 ± 1983 cpm and 22103 ± 1680 cpm respectively. There was also a statistically significant inhibition in thymidine incorporation from 20914 ± 1078 cpm in non-stimulated cells from CII/CFA sensitised mice to 10746 ± 618 and 7055 ± 395 cpm in non-stimulated cells isolated from leflunomide treated mice at 3 and 10mg/kg respectively.

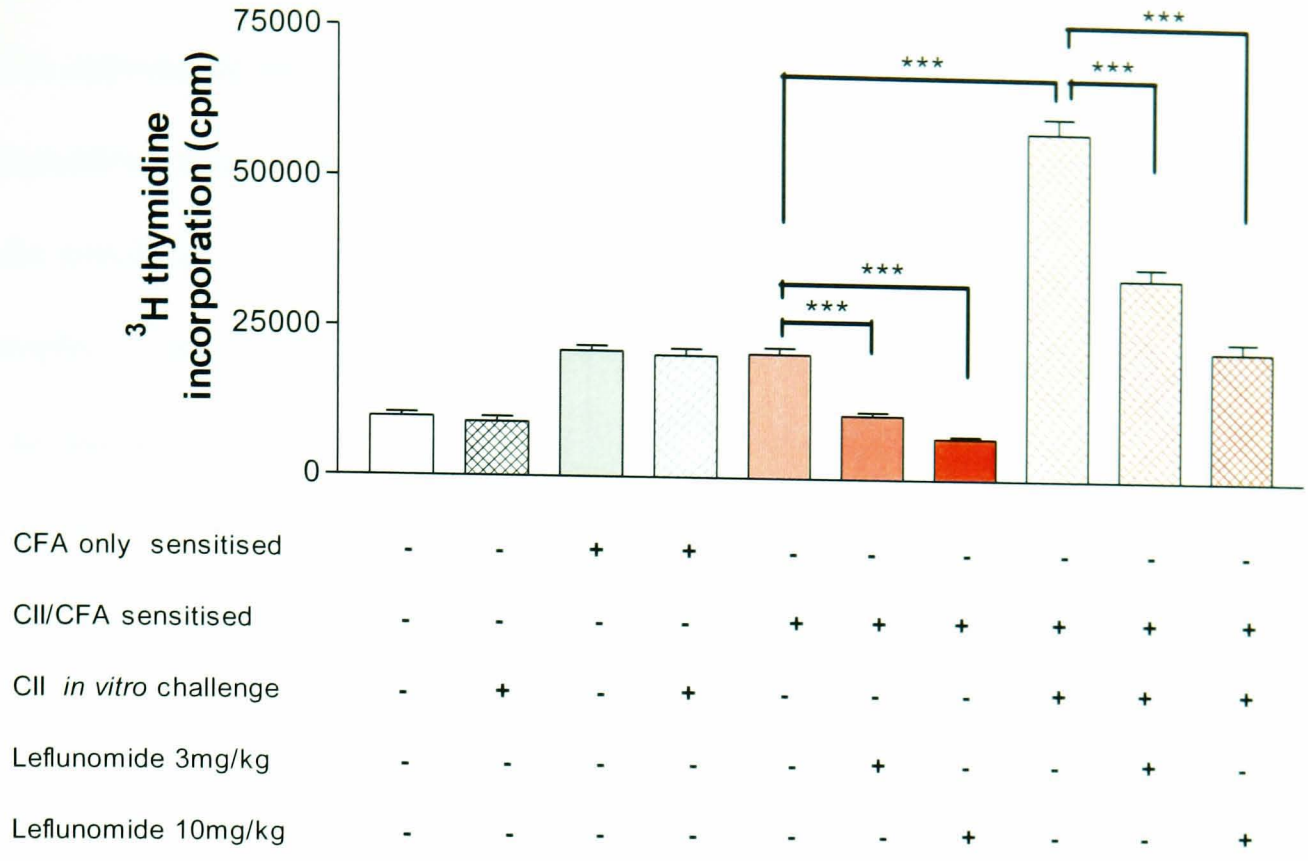


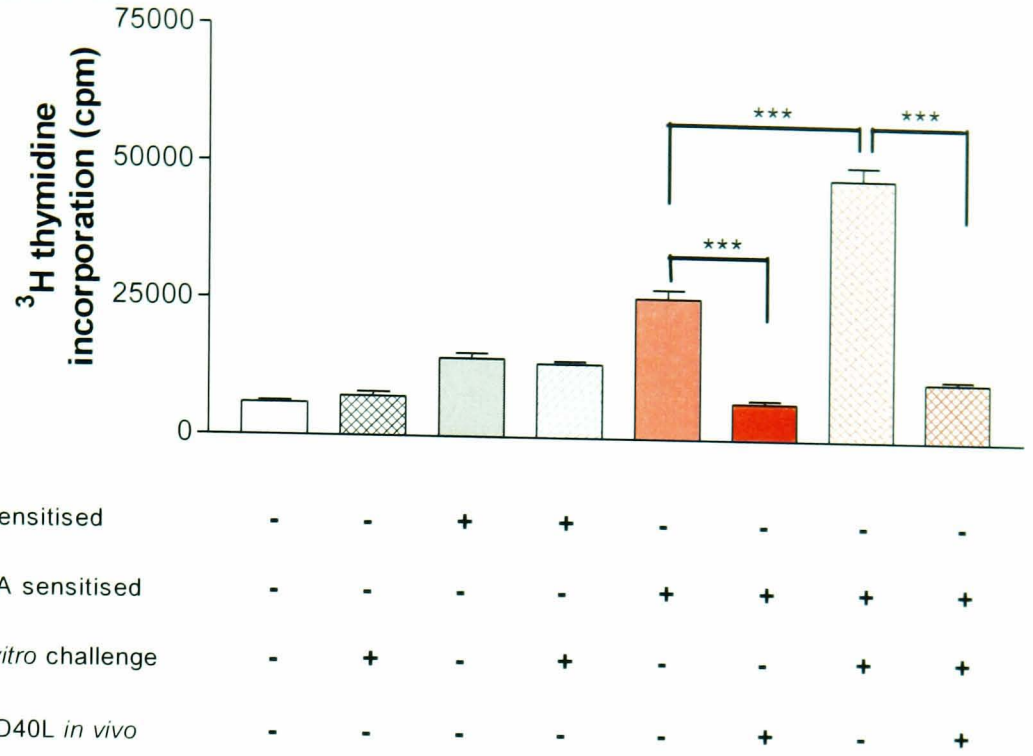
Figure 6.1 Effect of *in vivo* treatment with leflunomide on *ex vivo* collagen II stimulated thymidine incorporation.

$CD4^+$ T cells were prepared from inguinal lymph nodes of normal male DBA/1 mice or from mice 14 days after sensitisation with either complete Freund's adjuvant (CFA) or collagen II (CII) in CFA. Cells were plated out at 2.5 x 10^5 $CD4^+$ T cells together with 1 x 10^6 mitomycin C treated antigen presenting cells obtained from normal spleens. Some cells were stimulated with 50μg/ml denatured CII. Tritiated thymidine was added to the wells for the last 6 hours of culture and thymidine incorporation assessed as counts per minute (cpm). Some animals were dosed orally with leflunomide at 3 or 10mg/kg once a day from one day prior to sensitisation. Lymph nodes were pooled from n = 5-10 mice per group and cells were plated out in replicates of 5. Data presented as mean ± s.e.m, *** $p<0.001$ statistically significant from indicated control.

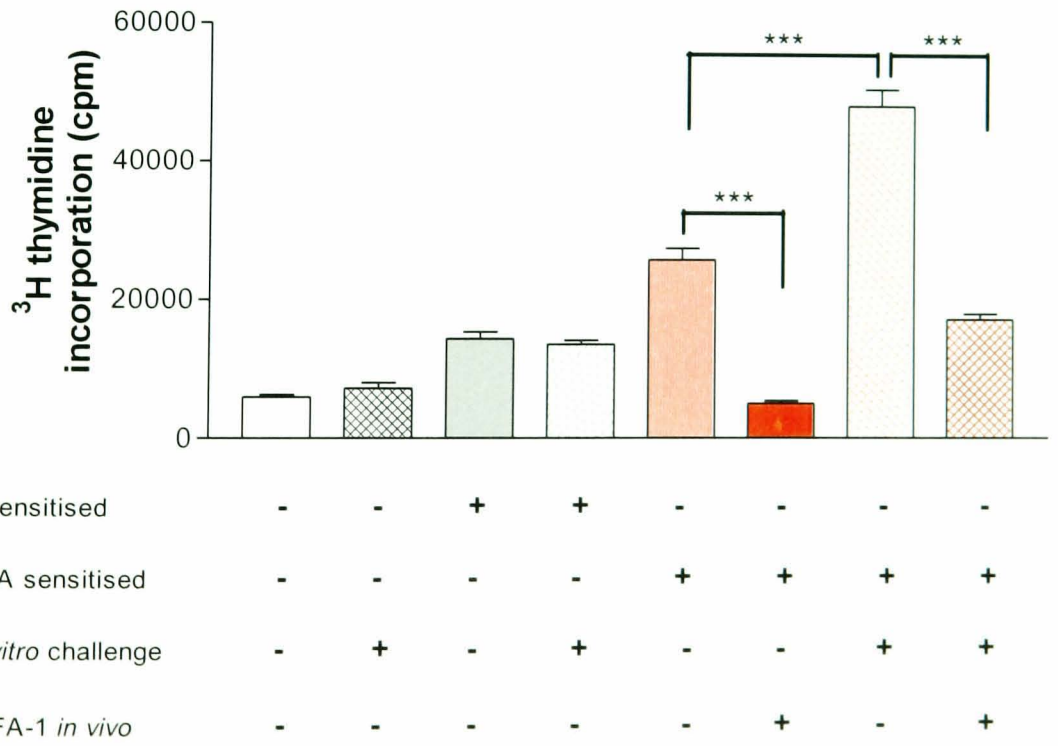
6.3.2 Anti-CD40L and anti-LFA-1

The importance of T cell – B cell and T cell – APC interactions in the $CD4^+$ T cell thymidine incorporation assay were investigated in the same study using anti-CD40L and anti-LFA-1 respectively (Figure 6.2). Data for each drug is presented in separate graphs. Control cells isolated from normal mice and CFA sensitised mice showed no increase in thymidine incorporation due to *in vitro* CII stimulation. However, there was still a slight increase between these two groups with non-stimulated cells from CFA sensitised mice increasing to 14391 ± 935 cpm as compared to 5904 ± 391 cpm in cells from normal mice. A further increase in thymidine incorporation to 25842 ± 1612 cpm was seen in non-stimulated cells from CII/CFA sensitised mice. When these cells were stimulated with CII *in vitro,* there was a statistically significant increase in thymidine incorporation to 48212 ± 2468 cpm. Treatment with anti-CD40L (Figure 6.2a) and anti-LFA-1 (Figure 6.2b) resulted in a statistically significant attenuation in this CII specific $CD4^+$ T cell thymidine incorporation to 10924 ± 521 cpm and 17167 ± 863 cpm respectively. In addition, both anti-CD40L and anti-LFA-1 caused a statistically significant inhibition in counts from 25842 ± 1612 cpm in non-stimulated cells from CII/CFA sensitised mice to 6758 ± 564 cpm and 4955 ± 480 cpm respectively in non-stimulated cells from drug treated mice.

a.


^{3}H thymidine incorporation (cpm)
75000
50000
25000
0
***
***
***
CFA sensitised - - + + - - - -
CII/CFA sensitised - - - - + + + +
CII *in vitro* challenge - + - + - - + +
Anti-CD40L *in vivo* - - - - - + - +

b.


^{3}H thymidine incorporation (cpm)
60000
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CFA sensitised - - + + - - - -
CII/CFA sensitised - - - - + + + +
CII *in vitro* challenge - + - + - - + +
Anti-LFA-1 *in vivo* - - - - - + - +

Figure 6.2 Effect of *in vivo* treatment with anti-CD40L and anti-LFA-1 antibodies on *ex vivo* collagen II stimulated thymidine incorporation.

$CD4^+$ T cells were prepared from inguinal lymph nodes of normal male DBA/1 mice or from mice 14 days after sensitisation with either complete Freund's adjuvant (CFA) or collagen II (CII) in CFA. Cells were plated out at 2.5 x 10^5 $CD4^+$ T cells together with 1 x 10^6 mitomycin C treated antigen presenting cells obtained from normal spleens. Some cells were stimulated with 50μg/ml denatured CII. Tritiated thymidine was added to the wells for the last 6 hours of culture and thymidine incorporation assessed as counts per minute (cpm). Some animals were dosed with anti-mouse CD40L (a) or anti-mouse LFA-1 (b) antibodies at 30mg/kg s.c. once a week from one day prior to sensitisation. Lymph nodes were pooled from n = 5-10 mice per group and cells were plated out in replicates of 5. Data presented as mean ± s.e.m, *** $p<0.001$ statistically significant from indicated control.

6.3.3 Anti-TNFα

To identify if the pro-inflammatory cytokine TNFα has a role to play in $CD4^+$ T cell proliferation, the anti-TNFα mAb was administered to mice sensitised to CII/CFA (Figure 6.3). In this study control cells isolated from normal mice gave counts of 1033 ± 69 cpm. Cells from CFA sensitised mice showed an increase in thymidine incorporation to 2549 ± 242 cpm. When these control cells were stimulated *in vitro* with CII there was no statistically significant increase in thymidine incorporation. Cells isolated from CII/CFA sensitised mice showed no increase in counts above that seen in cells from CFA sensitised mice. When these cells were stimulated with CII *in vitro* they showed a statistically significant increase in thymidine incorporation from 3283 ± 105 to 9395 ± 872 cpm. Treatment with anti-TNFα failed to inhibit this CII stimulated thymidine incorporation (11511 ± 1292 cpm).

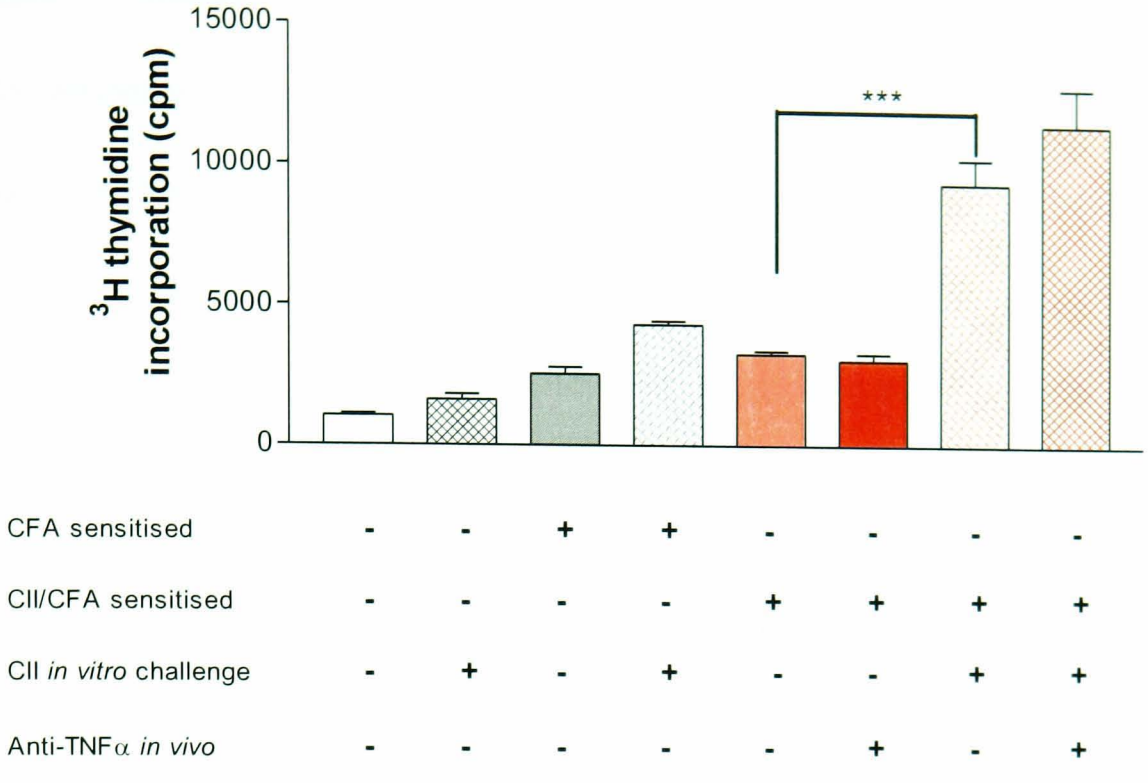


Figure 6.3 Effect of *in vivo* treatment with anti-TNFα antibody on *ex vivo* collagen II stimulated thymidine incorporation.

$CD4^+$ T cells were prepared from inguinal lymph nodes of normal male DBA/1 mice or from mice 14 days after sensitisation with either complete Freund's adjuvant (CFA) or collagen II (CII) in CFA. Cells were plated out at 2.5 x 10^5 $CD4^+$ T cells together with 1 x 10^6 mitomycin C treated antigen presenting cells obtained from normal spleens. Some cells were stimulated with 50μg/ml denatured CII. Tritiated thymidine was added to the wells for the last 6 hours of culture and thymidine incorporation assessed as counts per minute (cpm). Some animals were dosed with anti-mouse TNFα Fab PEG antibody at 100mg/kg s.c. twice a week from one day prior to sensitisation. Lymph nodes were pooled from n = 5-10 mice per group and cells were plated out in replicates of 5. Data presented as mean ± s.e.m, ** $p<0.01$ & *** $p<0.001$ statistically significant from indicated control.

6.3.4 Anti-IL-1β

To investigate the role of IL-1β in the CII stimulated $CD4^-$ T cell thymidine incorporation assay, the anti-IL-1β mAb was used (Figure 6.4). Control cells from normal mice and mice sensitised to CFA showed no response to *in vitro* CII stimulation. In this study there was no increase in counts between cells isolated from normal and CFA sensitised mice. Furthermore, non-stimulated cells from CII/CFA sensitised mice showed no increase in thymidine incorporation compared to control cells. However, when cells from CII/CFA sensitised mice were stimulated *in vitro* with CII there was a statistically significant increase in thymidine incorporation from 9446 ± 844 to 17690 ± 596 cpm. Anti-IL-1β treatment resulted in a statistically significant attenuation of CII stimulated $CD4^+$ T cell thymidine incorporation from 17690 ± 596 to 9986 ± 420 cpm. Furthermore, non-stimulated cells isolated from anti-IL-1β treated mice showed a statistically significant reduction in counts as compared to non-stimulated cells from CII/CFA sensitised mice, from 9446 ± 844 to 5375 ± 485 cpm.

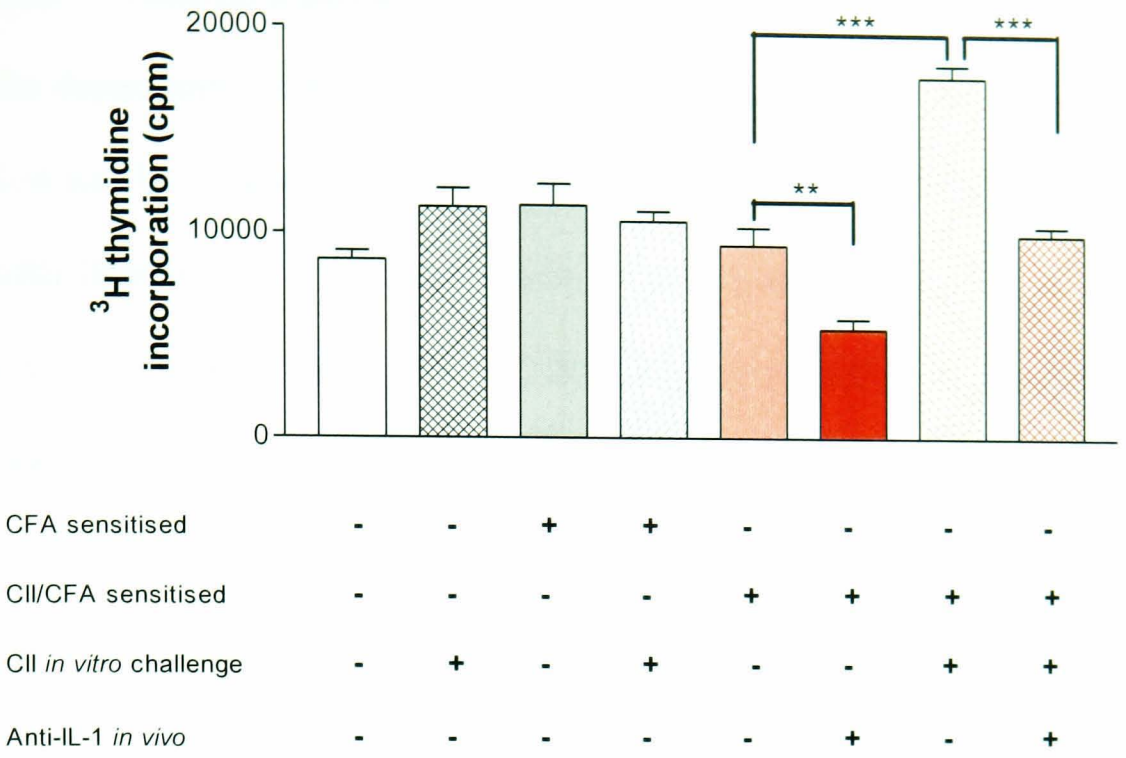


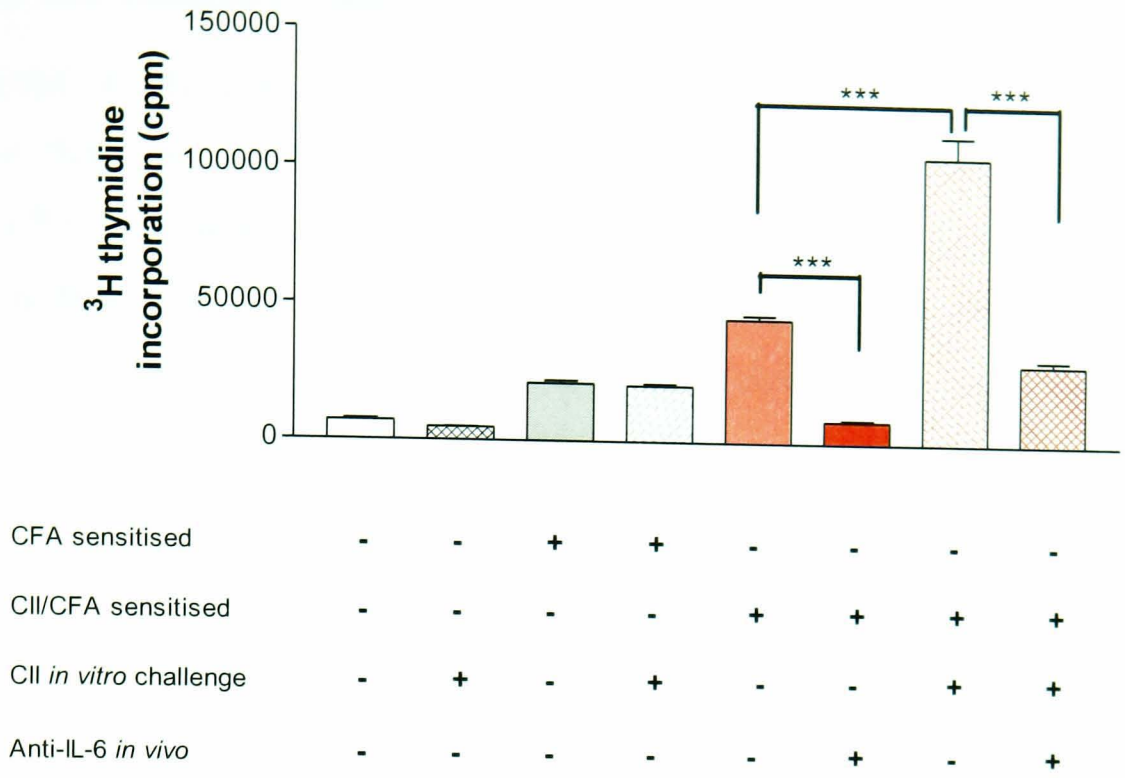
Figure 6.4 Effect of *in vivo* treatment with anti-IL-1β antibody on *ex vivo* collagen II stimulated thymidine incorporation.

$CD4^+$ T cells were prepared from inguinal lymph nodes of normal male DBA/1 mice or from mice 14 days after sensitisation with either complete Freund's adjuvant (CFA) or collagen II (CII) in CFA. Cells were plated out at 2.5 x 10^5 $CD4^+$ T cells together with 1 x 10^6 mitomycin C treated antigen presenting cells obtained from normal spleens. Some cells were stimulated with 50μg/ml denatured CII. Tritiated thymidine was added to the wells for the last 6 hours of culture and thymidine incorporation assessed as counts per minute (cpm). Some animals were dosed with anti-mouse IL-1β antibody at 10mg/kg s.c. once a week from one day prior to sensitisation. Lymph nodes were pooled from n = 5-10 mice per group and cells were plated out in replicates of 5. Data presented as mean ± s.e.m, ** $p<0.01$ & *** $p<0.001$ statistically significant from indicated control.

6.3.5 Anti-IL-6 and anti-IL-17

The dependency of the CII stimulated $CD4^+$ T cell thymidine incorporation assay on IL-6 and IL-17 was determined using the anti-IL-6 and anti-IL-17 mAbs in the same study (Figure 6.5). Moreover, anti-IL-17 was used to establish if this assay can detect activity of a novel drug. Data for each drug is presented in separate graphs. In this study there was an increase in thymidine incorporation between non-stimulated control cells isolated from normal and CFA sensitised mice. These counts were increased further in non-stimulated cells from CII/CFA sensitised mice. Control cells did not respond to *in vitro* CII stimulation. However, the addition of CII *in vitro* to cells from CII/CFA sensitised mice resulted in a statistically significant increase in thymidine incorporation from 45349 ± 1333 to 105320 ± 7771 cpm. Mice treated *in vivo* with anti-IL-6 (Figure 6.5a) and anti-IL-17 (Figure 6.5b) showed a statistically significant inhibition in CII stimulated thymidine incorporation, resulting in counts of 29467 ± 1824 and 29897 ± 1424 cpm respectively. In addition, anti-IL-6 and anti-IL-17 caused a statistically significant reduction in counts from 45349 ± 1333 in non-stimulated cells isolated from CII/CFA sensitised mice to 8360 ± 513 and 12152 ± 728 cpm respectively.

a.


150000
100000
50000
0
^{3}H thymidine incorporation (cpm)
***
***
***
CFA sensitised - - + + - - - -
CII/CFA sensitised - - - - + + + +
CII *in vitro* challenge - + - + - - + +
Anti-IL-6 *in vivo* - - - - - + - +

b.

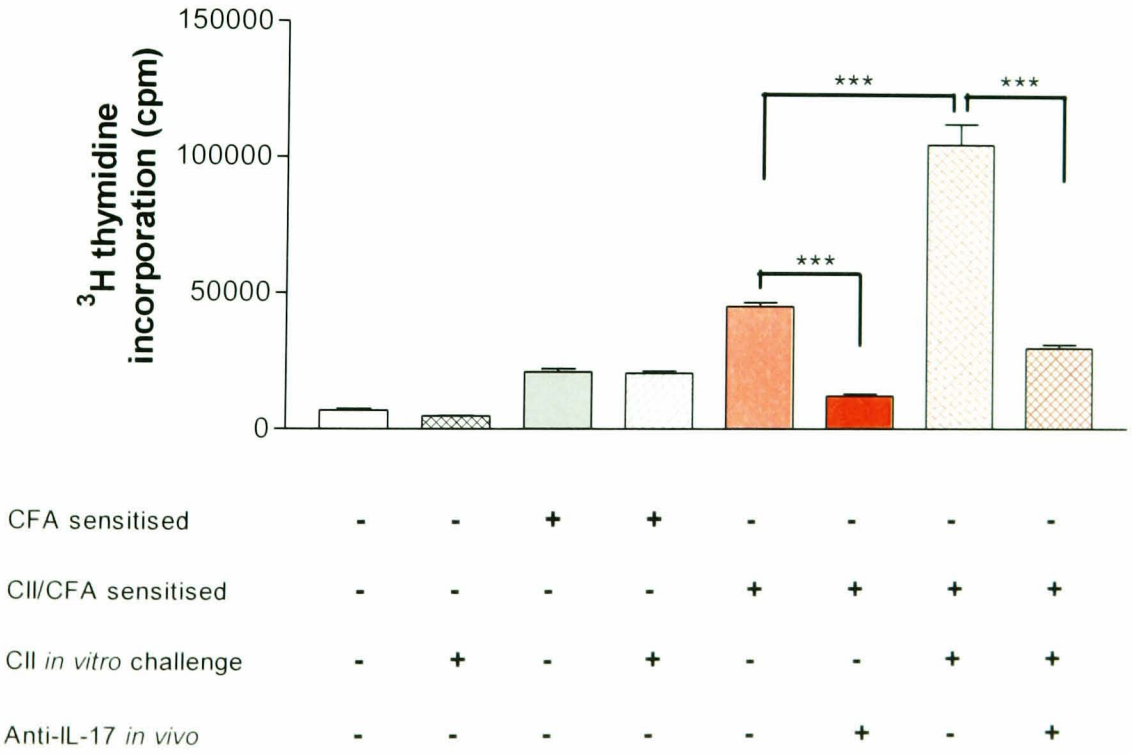

150000
100000
50000
0
^{3}H thymidine incorporation (cpm)
***
***
***
CFA sensitised - - + + - - - -
CII/CFA sensitised - - - - + + + +
CII *in vitro* challenge - + - + - - + +
Anti-IL-17 *in vivo* - - - - - + - +

Figure 6.5 Effect of *in vivo* treatment with anti-IL-6 and anti-IL-17 antibodies on *ex vivo* collagen II stimulated thymidine incorporation.

$CD4^+$ T cells were prepared from inguinal lymph nodes of normal male DBA/1 mice or from mice 14 days after sensitisation with either complete Freund's adjuvant (CFA) or collagen II (CII) in CFA. Cells were plated out at 2.5 x 10^5 $CD4^+$ T cells together with 1 x 10^6 mitomycin C treated antigen presenting cells obtained from normal spleens. Some cells were stimulated with 50μg/ml denatured CII. Tritiated thymidine was added to the wells for the last 6 hours of culture and thymidine incorporation assessed as counts per minute (cpm). Some animals were dosed with anti-mouse IL-6 (a) or anti-mouse IL-17 (b) antibodies at 10mg/kg s.c. once a week from one day prior to sensitisation. Lymph nodes were pooled from n = 5-10 mice per group and cells were plated out in replicates of 5. Data presented as mean ± s.e.m, *** $p<0.001$ statistically significant from indicated control.

6.3.6 Effect of in vitro drug administration on CII stimulated $CD4^+$ T cell thymidine incorporation

When cells were isolated to study the effects of *in vivo* drug administration on $CD4^+$ T cell thymidine incorporation, culture plates were also set up to assess the effect of drugs *in vitro* on $CD4^+$ T cells isolated from CII/CFA sensitised mice. Leflunomide could not be assessed *in vitro* as it is a pro-drug and the active constituent is a metabolite which is not commercially available. Instead cyclosporin, another anti-proliferative drug that has been used in the treatment of RA, was assessed. *In vitro* drug effects on CII stimulated thymidine incorporation are summarised in Table 6.1. Cyclosporin caused a statistically significant concentration dependent inhibition in CII stimulated $CD4^+$ T cell thymidine incorporation. Anti-LFA-1 and anti-IL-1β also caused a concentration dependent inhibition in CII stimulated $CD4^+$ T cell thymidine incorporation which became statistically significant at 1 and 10μg/mL. Anti-CD40L, anti-TNFα, anti-IL-6 and anti-IL-17 had no effect on CII stimulated $CD4^+$ T cell thymidine incorporation, when added *in vitro*.

Drug	Concentration *in vitro*	$CD4^+$ T cell thymidine incorporation
Cyclosporin	0.1, 1 and 10μM	+
Anti-CD40L	0.1, 1 and 10μg/mL	-
Anti-LFA-1	0.1μg/mL	-
	1 and 10μg/mL	+
Anti-TNFα	0.1, 1 and 10μg/mL	-
Anti-IL-1β	0.1μg/mL	-
	1 and 10μg/mL	+
Anti-IL-6	0.1, 1 and 10μg/mL	-
Anti-IL-17	0.1, 1 and 10μg/mL	-

Table 6.1 Summary of *in vitro* drug effects on *ex vivo* collagen II stimulated thymidine incorporation.

$CD4^+$ T cells isolated from *in vivo* studies were plated out at 2.5 x 10^5 $CD4^+$ T cells together with 1 x 10^6 mitomycin C treated antigen presenting cells obtained from normal spleens. Some cells were stimulated with 50μg/ml denatured CII. Tritiated thymidine was added to the wells for the last 6 hours of culture and thymidine incorporation assessed as counts per minute (cpm). Some cells isolated from CII/CFA sensitised mice and stimulated *in vitro* with CII received cyclosporin at a concentration of 0.1, 1 or 10μM. Other CII stimulated cells received anti-CD40L, anti-LFA-1, anti-TNFα, anti-IL-6 or anti-IL-17 at 0.1, 1 or 10μg/ml. Lymph nodes were pooled from n = 5-10 mice per group and cells were plated out in replicates of 5. Drugs were assessed for their ability to inhibit CII stimulated thymidine incorporation in cells from CII/CFA sensitised mice. + represents a statistically significant inhibition and – represents no effect.

6.4 Discussion

Table 6.2 summarises drug activity in the CIA model and CII stimulated $CD4^+$ T cell thymidine incorporation assay. The incorporation of thymidine in this assay was used as a marker of cell proliferation. It was assumed that the depletion of $CD4^+$ T cells would make this assay impractical therefore the anti-$CD4^+$ mAb was not assessed in this assay. In addition, the anti-$CD8^+$ mAb was not assessed as it has been demonstrated that $CD8^+$ T cells do not play a role in CIA or CII immunity (Chapters 3 and 5).

Drug	**CIA**	**$CD4^+$ T cell thymidine incorporation (*in vivo* drug effect)**	**$CD4^+$ T cell thymidine incorporation (*in vitro* drug effect)**
Leflunomide	+ **(68)**	+	**N/A**
Anti-CD40L	+ **(81)**	+	-
Anti-LFA-1	- **(38)**	+	+
Anti-TNFα	+ **(82)**	-	-
Anti-IL-1β	+ **(100)**	+	+
Anti-IL-6	+ **(87)**	+	-
Anti-IL-17	+ **(75)**	+	-

Table 6.2 Summary of drug effects on CIA and *ex vivo* CII stimulated thymidine incorporation.

Note: *+ represents a statistically significant inhibition in CIA or CII stimulated thymidine incorporation, as compared to control.*

- represents no change from control.

The numbers in brackets represent the percent reduction in the AUC of the clinical score as compared to controls.

Refer to Chapter 3 for CIA data.

In this chapter, leflunomide was used to validate the CII stimulated $CD4^+$ T cell thymidine incorporation assay. *In vivo* treatment with leflunomide caused a statistically significant inhibition in CII stimulated $CD4^+$ T cell thymidine incorporation. These data show that leflunomide is capable of inhibiting CII stimulated cell proliferation and confirm its reported anti-proliferative activity (Chong *et al.*, 1993). However, leflunomide could not be assessed *in vitro* in this assay. Instead the clinically relevant agent cyclosporin, which has been shown to inhibit CIA (Takagishi *et al.*, 1986) and is a known inhibitor of lymphocyte proliferation (Kasaian and Biron, 1990), was assessed *in vitro*. In the current study its anti-proliferative activity was confirmed by its ability to inhibit CII stimulated thymidine incorporation into $CD4^+$ T cells.

The effects of these anti-proliferative drugs on thymidine incorporation support this readout as a marker of proliferation. Furthermore, these data confirm the importance of $CD4^+$ T cells in CII immunity and demonstrated that clinically relevant drugs are active in this pre-arthritic readout.

The underlying immune response to CII in this assay was investigated using anti-CD40L and anti-LFA-1 mAbs. In the current study, *in vivo* anti-CD40L treatment caused a total abrogation of CII stimulated $CD4^+$ T cell proliferation. These data demonstrate the importance of T cell – B cell interactions in CII immunity and confirm the role of this interaction in T cell proliferation as reported by Cayabyab *et al.* (1994). Furthermore, these data support findings in the hypersensitivity model (Chapter 5) which suggested CD40L may have a role to play in T cell activation. However, when anti-CD40L was added to cell cultures *in vitro* it failed to inhibit cell proliferation (Table 6.1).

The administration of anti-LFA-1, both *in vivo* and *in vitro*, caused a statistically significant inhibition in CII stimulated $CD4^+$ T cell proliferation. This demonstrates that T cell – APC interactions are essential in the cellular immune response to CII in the pre-arthritic phase and confirms a role for LFA-1, as suggested in Chapter 5, in these short-term readouts. It is surprising that such a profound suppression on $CD4^+$ T cell proliferation with anti-LFA-1 did not translate into a statistically significant inhibition in CIA. This further supports the theory that LFA-1 is an essential integrin in the initiation of CII immunity but less important as disease progresses. This suggests that LFA-1 may not be the only integrin to play a role in T cell – APC interactions.

The role of TNFα, IL-1β, IL-6 and IL-17 in this assay was investigated using mAbs that target these cytokines. *In vivo* administration of anti-IL-1β, anti-IL-6 and anti-IL-17, but not anti-TNFα, had profound effects on CII stimulated $CD4^+$ T cell proliferation. However, it was only the addition of anti-IL-1β *in vitro* that caused a statistically significant inhibition in cell proliferation.

These data confirm findings in Chapter 5 which suggested a lack of effect of anti-TNFα on the cellular immune response to CII. In support of these findings, Campbell *et al.* (2001) showed that TNF deficient mice have normal T cell proliferation in response to CII. The anti-arthritic effect of anti-TNFα therefore appears not to be dependent on inhibition of $CD4^+$ T cell proliferation. However, it does suggest that the anti-arthritic effects of anti-IL-1β, anti-IL-6 and anti-IL-17 may be mediated in part through their inhibitory action on $CD4^+$ T cell proliferation. Furthermore, the inhibitory effect of anti-IL-1β on cell proliferation *in vitro* suggests it may be capable of influencing an established immune response as well as the generation of CII

specific cells *in vivo*. Whereas it appears anti-IL-6 and anti-IL-17 only inhibit the generation of CII specific T cells and have no effect on an established response. These data confirm work conducted in CIA models by Saijo *et al.* (2002) and Takagi *et al.* (1998) who identified a role for IL-1 and IL-6 respectively in CII stimulated cell proliferation. However, it would appear that this is the first time that mAbs against IL-1β, IL-6 and IL-17 have been shown to inhibit CII stimulated $CD4^+$ T cell proliferation in pre-arthritic mice. These data have further validated this short-term readout of CII immunity and shown the assay to be IL-1β, IL-6 and IL-17 dependent. In addition, it appears this assay is capable of detecting the activity of novel therapies such as anti-IL-17.

Data in Chapter 5 suggested that leflunomide, anti-CD40L, anti-LFA-1, anti-IL-1β, anti-IL-6 and anti-IL-17 may have effects on the cellular immune response to CII as indicated by their ability to suppress CII induced hypersensitivity at 24 hours. This chapter has demonstrated that these drugs also suppress $CD4^+$ T cell proliferation thereby confirming their role in cellular immunity. These data also suggest that the 24 hour hypersensitivity time point can be used as a readout of cellular immunity.

In addition, this assay has shown that in response to CFA sensitisation there appears to be an increase in non-stimulated $CD4^+$ T cell proliferation as compared to cells from normal mice. This response seems to be enhanced further when mice have been sensitised to CII/CFA. This suggests, *in vivo*, that $CD4^+$ T cells are proliferating in response to adjuvant and CII prior to being re-stimulated. Drugs that inhibited CII stimulated $CD4^+$ T cell proliferation also caused a statistically significant reduction in this antigen independent proliferation, suggesting they suppress the initial cellular response to sensitisation.

Most of these drugs had profound anti-proliferative effects. However, it appears that cells isolated from drug treated mice can still proliferate in response to CII *ex vivo*, albeit at a much lower magnitude than cells from CII/CFA sensitised mice. This proliferative response was statistically significant in cells isolated from leflunomide, anti-LFA-1 ($P<0.001$), anti-IL-1β, anti-IL-6 and anti-IL-17 ($P<0.01$) treated mice (statistical significance not indicated on graphs). This suggests, even in the presence of a drug, there are a small proportion of cells capable of responding to CII. However, it appears that these cells are not able to elicit CIA. Anti-CD40L was the only drug that prevented this statistically significant increase in proliferation suggesting the interaction between T cells and B cells *in vivo* is vitally important in the generation of CII specific $CD4^+$ T cells.

The data summarised in Table 6.2 showed, in general, that drugs which suppressed CII stimulated $CD4^+$ T cell proliferation, after *in vivo* administration, also inhibit CIA, therefore demonstrating this assay is predictive of anti-arthritic drug activity. However, anti-LFA-1 was an exception as it caused a statistically significant reduction in cell proliferation but failed to suppress CIA. The effect of anti-LFA-1 in this short-term readout of CII immunity, like its effects on the other readouts in Chapter 5, represents a false positive result. However, this is better than having a false negative result where active anti-arthritic drugs may be overlooked. This is of much more concern and in this assay anti-TNFα gave a false negative result as it had no effect on cell proliferation but did suppress CIA. This assay should therefore not be used in isolation.

The lack of effect seen with anti-TNFα in the current study does however suggest that this assay may be able to discriminate between drugs based on their mechanism of action.

The assessment of drugs *in vitro* was unable to predict the anti-arthritic activity of a number of drugs. However, it may be of value when trying to differentiate drugs mechanistically.

In this chapter the CII stimulated $CD4^+$ T cell thymidine incorporation assay has been characterised and validated as another short-term readout of CII immunity that is predictive of drug effects in CIA. It has also been demonstrated that anti-IL-17 may, at least in part, exert its anti-arthritic activity in the CIA model by inhibiting $CD4^+$ T cell proliferation. These results suggest that IL-17 is a key cytokine in CII stimulated $CD4^+$ T cell biology. The role of IL-17 in T cell biology in this model is currently not well defined, the next chapter therefore sought to investigate this further.

Chapter seven

The role of IL-17 in CII immunity

7.1 Introduction

So far this thesis has demonstrated that CIA and CII immunity are dependent on $CD4^+$ T cells, with depletion of these cells resulting in the inhibition of disease, CII-induced hypersensitivity and anti-CII Ab production. Furthermore, it has been demonstrated that $CD4^+$ T cells isolated from CII/CFA sensitised mice proliferate in response to *in vitro* CII stimulation. However, the relative involvement of the different $CD4^+$ T cell subsets in CII immunity could not be determined using the proliferation assay.

Research has shown that cells cultured from CIA mice produce IFNγ and IL-4 in response to *in vitro* CII stimulation indicating the presence of Th1 and Th2 $CD4^+$ T cell responses respectively (Mauri *et al.*, 1996 and Doncarli *et al.*, 1997). In these studies, cytokine analysis at various time points during CIA suggested the temporal involvement of Th1 and Th2 cells in disease progression and resolution respectively.

The recent identification of a population of $CD4^+$ IL-17 producing T cells, termed "Th17 cells", that are distinct from Th1 and Th2 cells (Harrington *et al.*, 2005) has brought into focus a shift in this cellular paradigm. However, the role of IL-17 secreting Th17 cells in relation to IFNγ secreting Th1 and IL-4 secreting Th2 cells in the CIA model has not been defined.

In this chapter the relative involvement of Th1, Th2 and Th17 cells during disease progression was inferred by measuring IFNγ, IL-4 and IL-17 respectively. The production of these cytokines in response to CII was assessed in supernatants from $CD4^+$ T cell cultures by ELISA. In addition, cytokine specific enzyme-linked immunospot (ELISpot) assays were utilised. ELISpot assays detect cytokines produced by cells in culture as spots and one spot is thought to represent a single cytokine secreting cell. These assays were used to determine the frequency of IFNγ,

IL-4 and IL-17 positive spots produced in CII stimulated $CD4^+$ T cell cultures, something that could not be determined by ELISA.

The relationship between IFNγ, IL-4 and IL-17 was then investigated in these cell cultures by adding anti-IFNγ, anti-IL-17, recombinant mouse (rm) IFNγ, rmIL-4 and rmIL-17 to different cytokine specific ELISpot plates.

The cytokine network involved in CII immunity was investigated further using the anti-IL-6 Ab. This research arose because IL-6 was recently shown to support the differentiation of naïve $CD4^+$ T cells into IL-17 producing T cells (Veldhoen *et al.*, 2006). However, its role in IL-17 production in CII immunity has not been investigated. The effect of *in vivo* anti-IL-6 treatment on *ex vivo* IL-17 production from $CD4^+$ T cells was therefore assessed in this chapter. In addition, the effect of *in vivo* anti-IL-17 treatment on *ex vivo* IL-17 production was also investigated.

7.2 Aim

The aim of this chapter was to investigate the role of IL-17 in CII immunity and investigate the relationship that may exist between Th17, Th1 and Th2 cells.

7.3 Results

In the following set of experiments CII stimulated $CD4^+$ T cell cultures were set up. In some studies IL-17, IFNγ and IL-4 production was assessed in cell culture supernatants by ELISA and quantified as picograms per millilitre (pg/mL). In other studies the frequency of IL-17, IFNγ and IL-4 positive spots was determined by ELISpot. To assess CII stimulated $CD4^+$ T cell proliferation the incorporation of tritiated thymidine into cells was analysed and measured as cpm. For a detailed description of the ELISA and ELISpot protocols refer to Chapter 2. Data is presented as the mean per group and statistical analysis of the data was conducted by one-way ANOVA with Bonferroni as the post-test, $p<0.05$ was considered statistically significant.

7.3.1 Time course of IL-17, IFNγ and IL-4 production in the CII stimulated $CD4^+$ T cell assay

In order to identify the time course of cytokine production a CII stimulated $CD4^+$ T cell assay was set up. Cell culture supernatants were analysed over a 96 hour culture period for the presence of IL-17, IFNγ and IL-4 (Figure 7.1), which are indicative of Th17, Th1 and Th2 cell responses respectively.

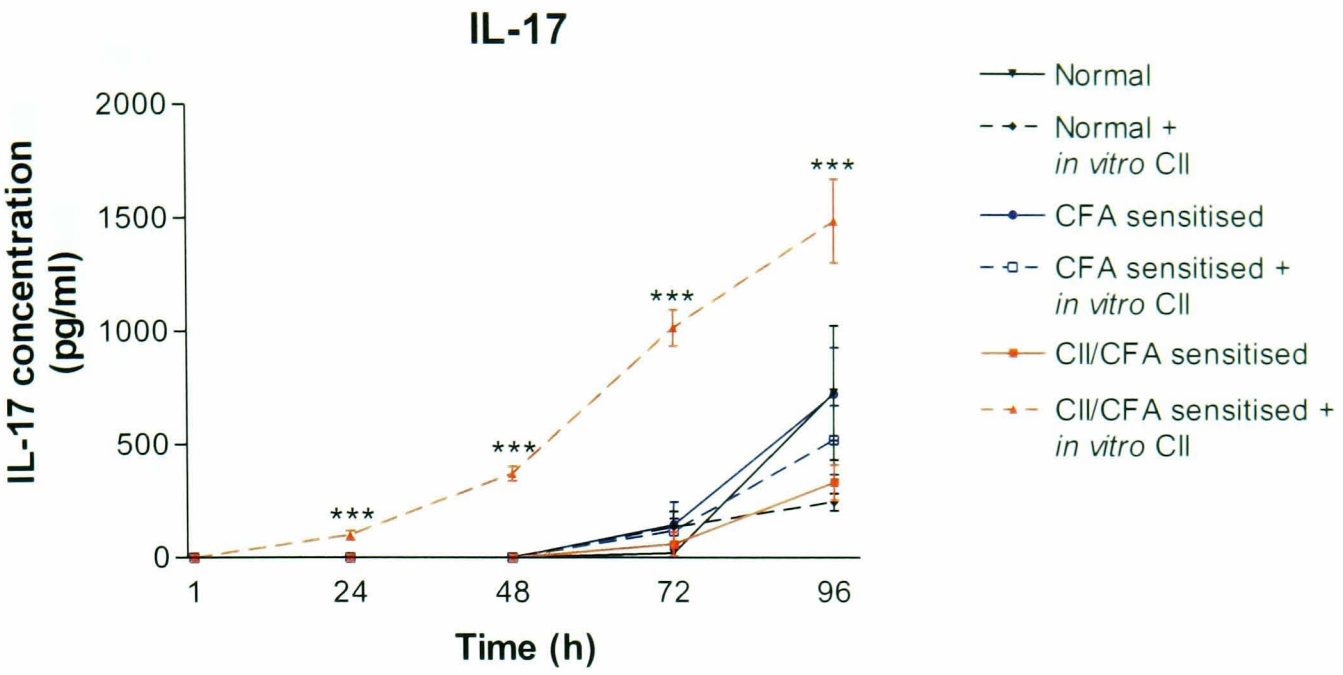
$CD4^+$ T cells isolated from normal mice and mice sensitised to CFA were used as controls. There was no statistically significant change in IL-17, IFNγ or IL-4 levels in supernatants from control cells over time. Furthermore, *in vitro* CII stimulation of these cells did not cause any statistically significant increase in IL-17, IFNγ or IL-4 levels above that of non-stimulated cells (Figure 7.1a, b and c). Cells isolated from CII/CFA sensitised mice showed no statistically significant increase in IL-17, IFNγ or IL-4 levels over the culture period as compared to CFA control cells. However, when

cells from CII/CFA sensitised mice were stimulated with CII *in vitro* they produced a statistically significant increase in IL-17 as compared to non-stimulated cells at 24 to 96 hours of culture. In addition, the amount of IL-17 produced from these cells increased over time (100 ± 21, 372 ± 31, 1019 ± 80 and 1491 ± 184 pg/ml at 24, 48. 72 and 96 hours respectively, Figure 7.1a).

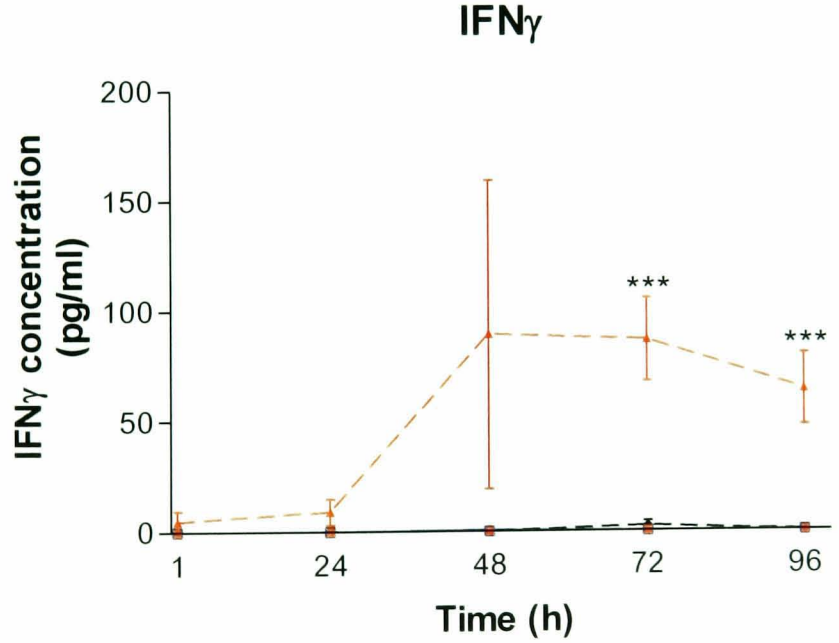
Assessment of IFNγ levels in the same samples showed that cells isolated from CII/CFA sensitised mice did not increase their production in response to *in vitro* CII stimulation at 1 or 24 hours of culture. At 48 hours of culture IFNγ was detected in culture supernatants from these cells (90 ± 70 pg/ml) but this was not statistically significant compared to non-stimulated cells. However, the production of IFNγ in response to *in vitro* CII was statistically significant at 72 hours (87 ± 19pg/ml) and 96 hours (64 ± 16 pg/ml) of culture as compared to non-stimulated cells (Figure 7.1b).

Further assessment of these samples showed that *in vitro* CII stimulation of cells from CII/CFA sensitised mice did not increase IL-4 production above that of non-stimulated cells at any time point (Figure 7.1c).

a.


IL-17
IL-17 concentration (pg/ml)
2000
1500
1000
500
0
1
24
48
72
96
Time (h)
***
***
***
Normal
Normal + in vitro CII
CFA sensitised
CFA sensitised + in vitro CII
CII/CFA sensitised
CII/CFA sensitised + in vitro CII

b.


IFNγ
IFNγ concentration (pg/ml)
200
150
100
50
0
1
24
48
72
96
Time (h)
***
***

c.

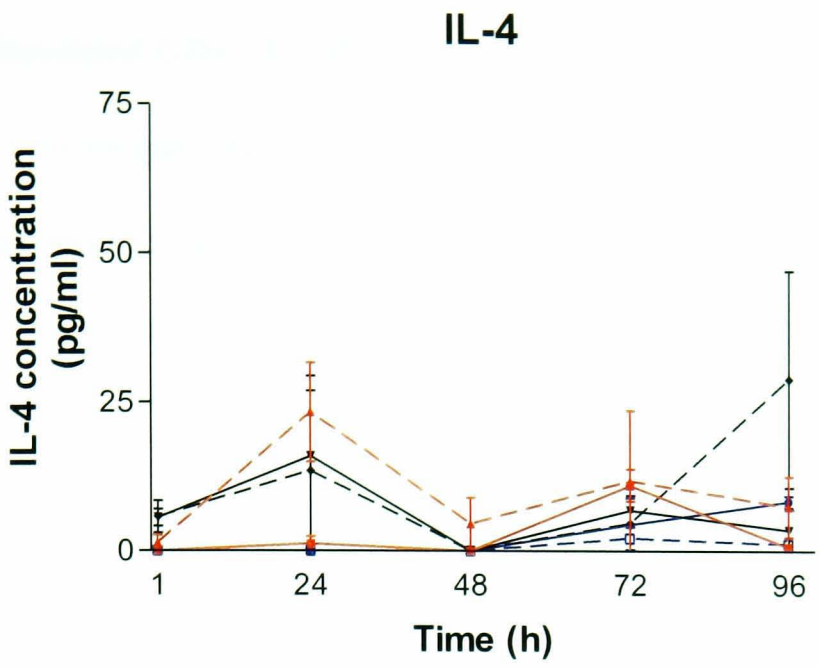


Figure 7.1 Time course of IL-17, IFNγ and IL-4 production in the collagen II stimulated $CD4^+$ T cell assay.

$CD4^+$ T cells were prepared from inguinal lymph nodes of normal male DBA/1 mice or from mice 14 days after sensitisation with either complete Freund's adjuvant (CFA) or collagen II (CII) in CFA. Cells were plated out at 2.5 x 10^5 $CD4^+$ T cells together with 1 x 10^6 mitomycin C treated antigen presenting cells obtained from normal spleens. Some wells were stimulated with 50μg/mL denatured CII. Cell supernatants were removed at 1, 24, 48, 72 and 96 hours of culture and mouse IL-17 (a), IFNγ (b) and IL-4 (c) levels assessed by ELISA (R&D Systems). Lymph nodes were pooled from n = 5-10 mice per group and cells were plated out in replicates of 5. Data presented as mean ± s.e.m, *** $p<0.001$ statistically significant difference as compared to non-stimulated cells from CII/CFA sensitised mice.

7.3.2 Investigation into the relationship between IL-17 and IFNγ in the CII stimulated CD4$^+$ T cell assay

To investigate the relationship between IL-17 and IFNγ in CII stimulated CD4$^+$ T cell cultures, ELISpot assays were utilised. As in previous experiments cell concentrations of 2.5 x 10^5 CD4$^+$ and 1 x 10^6 APCs were cultured in the presence or absence of CII and the frequency of IL-17 and IFNγ positive spots were assessed by ELISpot.

The addition of CII *in vitro* to cells from CII/CFA sensitised mice resulted in wells that were too dark to count and no discrete spot formation could be identified for either IL-17 or IFNγ at 24 and 72 hours of culture (historical data). In order to obtain a quantifiable response, further studies were conducted using a range of CD4$^+$ and APC concentrations (historical data). Results from theses studies indicated that a concentration of 2.5 x 10^5 CD4$^+$ T cells cultured with a reduced APC concentration of 1 x 10^5 in response to CII stimulation produced quantifiable IL-17 and IFNγ positive spots.

In order to show that this APC concentration, which has not been previously used in culture, was capable of stimulating CD4$^+$ T cells, a thymidine incorporation assay was set up (Figure 7.2). In this assay, control cells did not respond to *in vitro* CII stimulation and showed similar levels of thymidine incorporation consistent with previous work (Chapter 6). CD4$^+$ T cells isolated from CII/CFA sensitised mice showed a similar level of thymidine incorporation as controls (6652 ± 1181 cpm). *In vitro*, CII stimulation of these cells caused a statistically significant increase in thymidine incorporation (12128 ± 1443 cpm). These data show that APCs at a concentration of 1 x 10^5 are sufficient to elicit a proliferative response to CII which is similar to that seen when using a concentration of 1 x 10^6 APCs (Chapter 6).

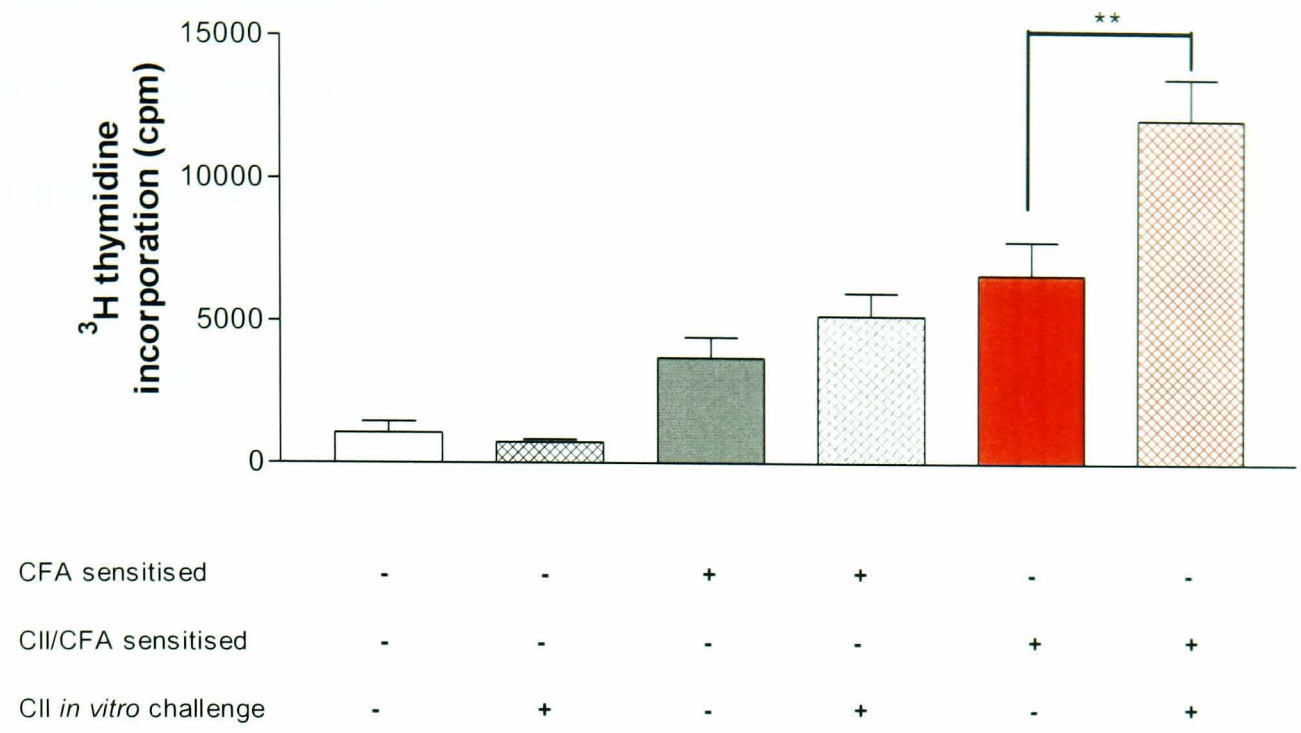


Figure 7.2 *Ex vivo* collagen II stimulated thymidine incorporation.

$CD4^+$ T cells were prepared from inguinal lymph nodes of normal male DBA/1 mice or from mice 14 days after sensitisation with either complete Freund's adjuvant (CFA) or collagen II (CII) in CFA. Cells were plated out at 2.5 x 10^5 $CD4^+$ T cells together with 1 x 10^5 mitomycin C treated antigen presenting cells obtained from normal spleens. Some wells were stimulated with 50μg/ml denatured CII. Tritiated thymidine was added to the wells for the last 6h of culture and thymidine incorporation assessed as counts per minute (cpm). Lymph nodes were pooled from n = 5-10 mice per group and cells were plated out in replicates of 5. Data presented as mean ± s.e.m, ** $p<0.01$ statistically significant from indicated control.

These culture conditions were then used in ELISpot assays to analyse the frequency of IL-17 and IFNγ positive spots (Figure 7.3). In these assays, low levels of IL-17 (Figure 7.3a and b) and IFNγ (Figure 7.3c and d) positive spots were detected in wells from control cells isolated from normal mice and CFA sensitised mice at 24 and 72 hours of culture. CII stimulation of these control cells did not increase the number of positive spots. There was, however, a slight increase in the number of IL-17 and IFNγ positive spots in these controls over time, but this was not statistically significant.

When cells from CII/CFA sensitised mice were cultured there was no change above control levels in the number of IL-17 positive spots at 24 or 72 hours (9.80 ± 1.80 and 23.40 ± 0.93 positive spots respectively). Stimulation of these cells with CII *in vitro* resulted in a statistically significant increase in the number of IL-17 positive spots at 24 and 72 hours (27.20 ± 2.08 and 44.40 ± 2.29 respectively). These data also show that there is an increase in the number of IL-17 positive spots over time. The relationship between IFNγ and IL-17 was investigated in this assay by using an anti-IFNγ mAb which was added to some wells (Figure 7.3a and b). Anti-IFNγ at a concentration of 1μg/ml caused a slight increase in the number of CII stimulated IL-17 positive spots at 24 and 72 hours (38.00 ± 4.66 and 52.00 ± 3.41 positive spots respectively) as compared to CII stimulated cells from CII/CFA sensitised mice. However, this was not statistically significant. Anti-IFNγ at 10 and 100μg/ml had no effect on the number of IL-17 positive spots produced in response to CII at 24 or 72 hours of culture.

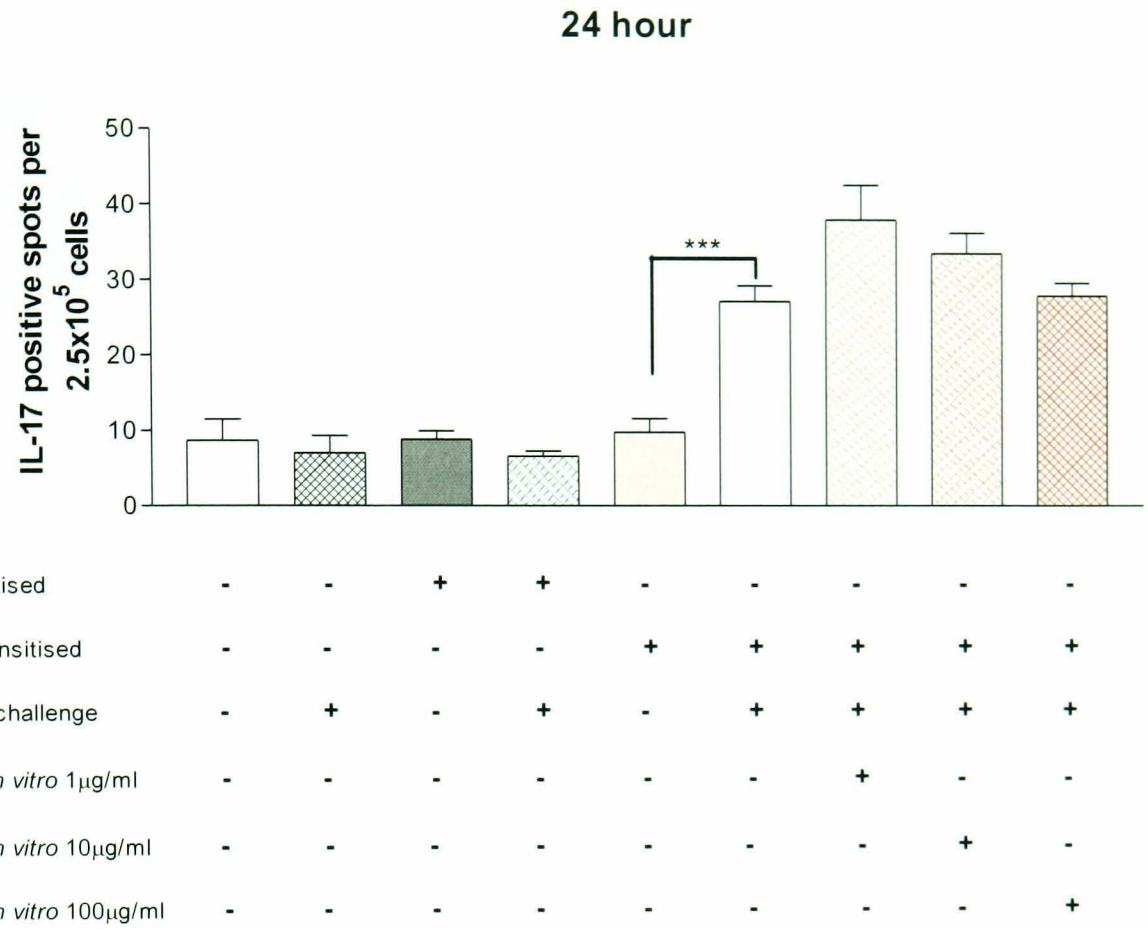
Assessment showed that IFNγ positive spots in cultures of cells from CII/CFA sensitised mice were consistent with those levels seen in controls at 24 and 72 hours (0.60 ± 0.25 and 0.20 ± 0.20 positive spots respectively). However, when these cells

were stimulated with CII there was an increase in the number of IFNγ positive spots at 24 hours (4.60 ± 0.75 positive spots). This response became statistically significant at 72 hours of culture (12.00 ± 1.05 positive spots). These data also indicate that the number of CII stimulated IFNγ positive spots increase over time. In these cultures the effect of blocking IL-17 on the number of IFNγ positive spots was assessed using anti-IL-17. In cultures of CII stimulated cells isolated from CII/CFA sensitised mice, anti-IL-17 caused an increase in the frequency of IFNγ positive spots at a concentration of 1, 10 and 100μg/ml after 24 hours of culture (8.00 ± 0.32, 9.80 ± 2.29 and 10.60 ± 1.327 positive spots respectively). This response was statistically significant at 10 and 100μg/ml as compared to CII stimulated cells from CII/CFA sensitised mice.

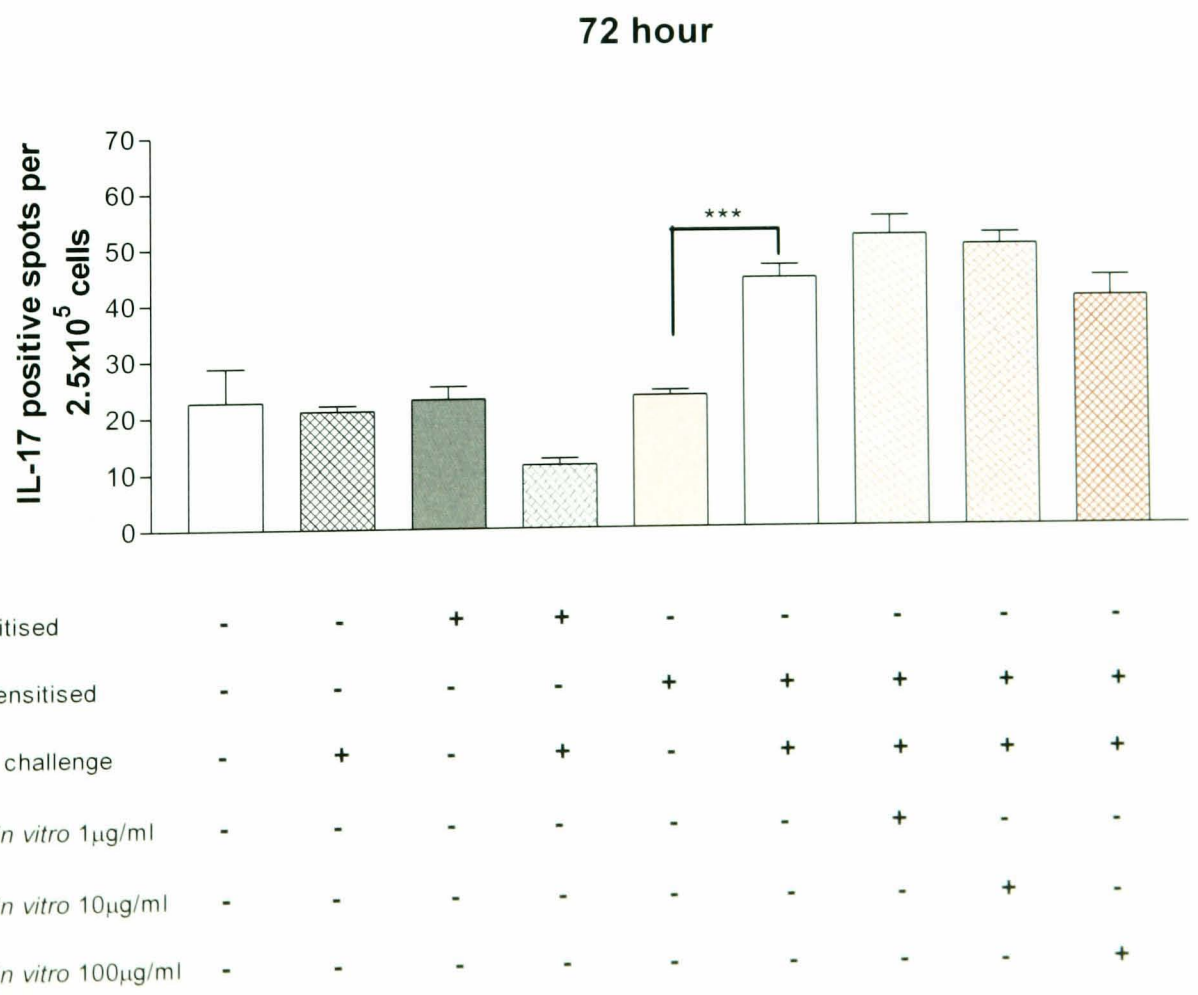
At 72 hours of culture anti-IL-17 at 1μg/ml increased the number of CII stimulated IFNγ positive spots (15.20 ± 2.52 positive spots), however this was not statistically significant. Unfortunately, anti-IL-17 at 10 and 100μg/ml resulted in wells that were too dark to distinguish individual IFNγ positive spots (Figure 7.3e).

These data suggest that anti-IL-17 increases the frequency of IFNγ positive spots in a concentration dependent manner. In a previous study, where a reduced $CD4^+$ T cell concentration was used that was not capable of responding to CII, anti-IL-17 at 100μg/ml had no effect on the number of IFNγ positive spots (data not shown), indicating that it is not the antibody itself affecting the ELISpot readout.

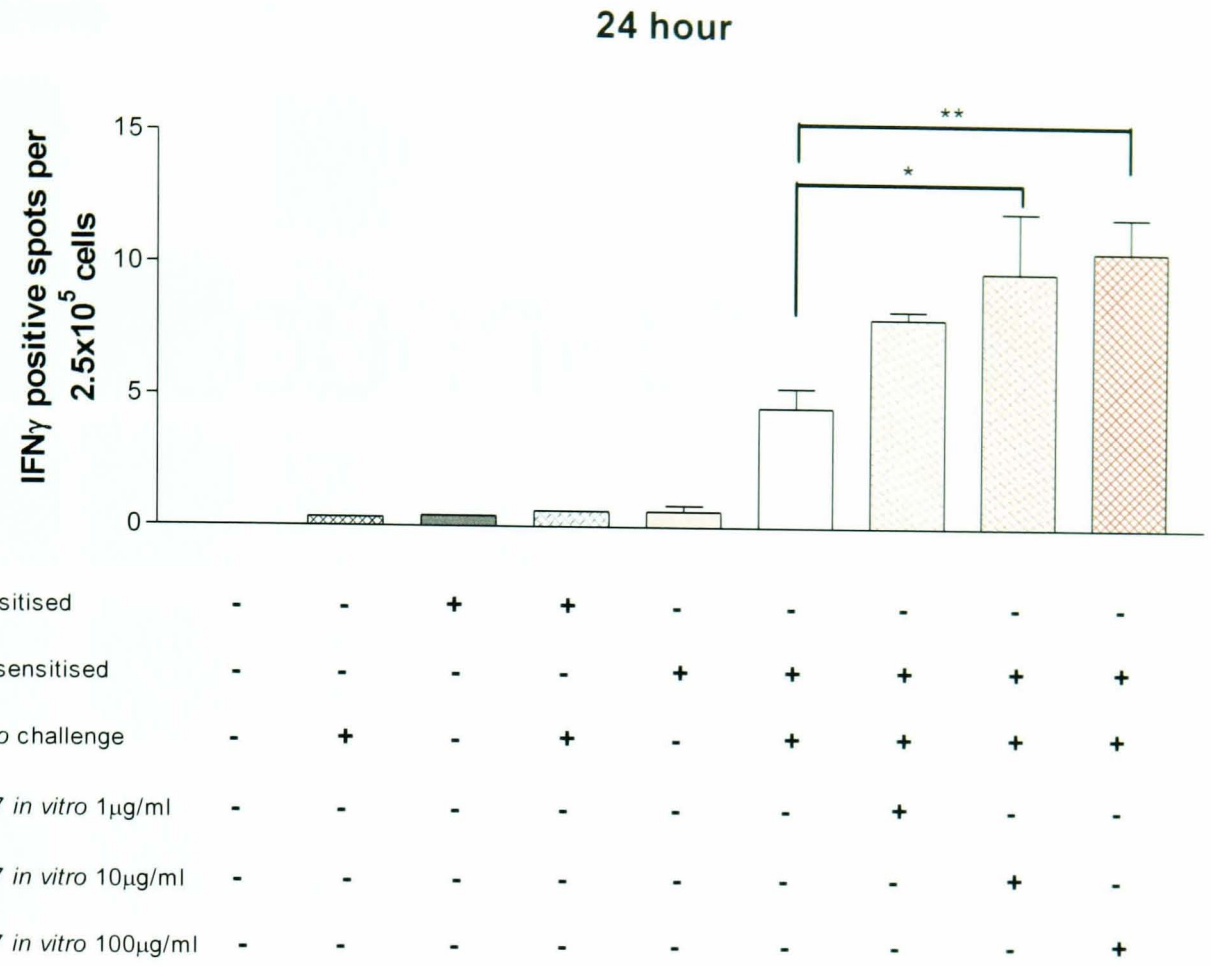
a.


24 hour
IL-17 positive spots per 2.5x10^5 cells
50
40
30
20
10
0
***
CFA sensitised - - + + - - - - -
CII/CFA sensitised - - - - + + + + +
CII in vitro challenge - + - + - + + + +
Anti-IFNγ in vitro 1μg/ml - - - - - - + - -
Anti-IFNγ in vitro 10μg/ml - - - - - - - + -
Anti-IFNγ in vitro 100μg/ml - - - - - - - - +

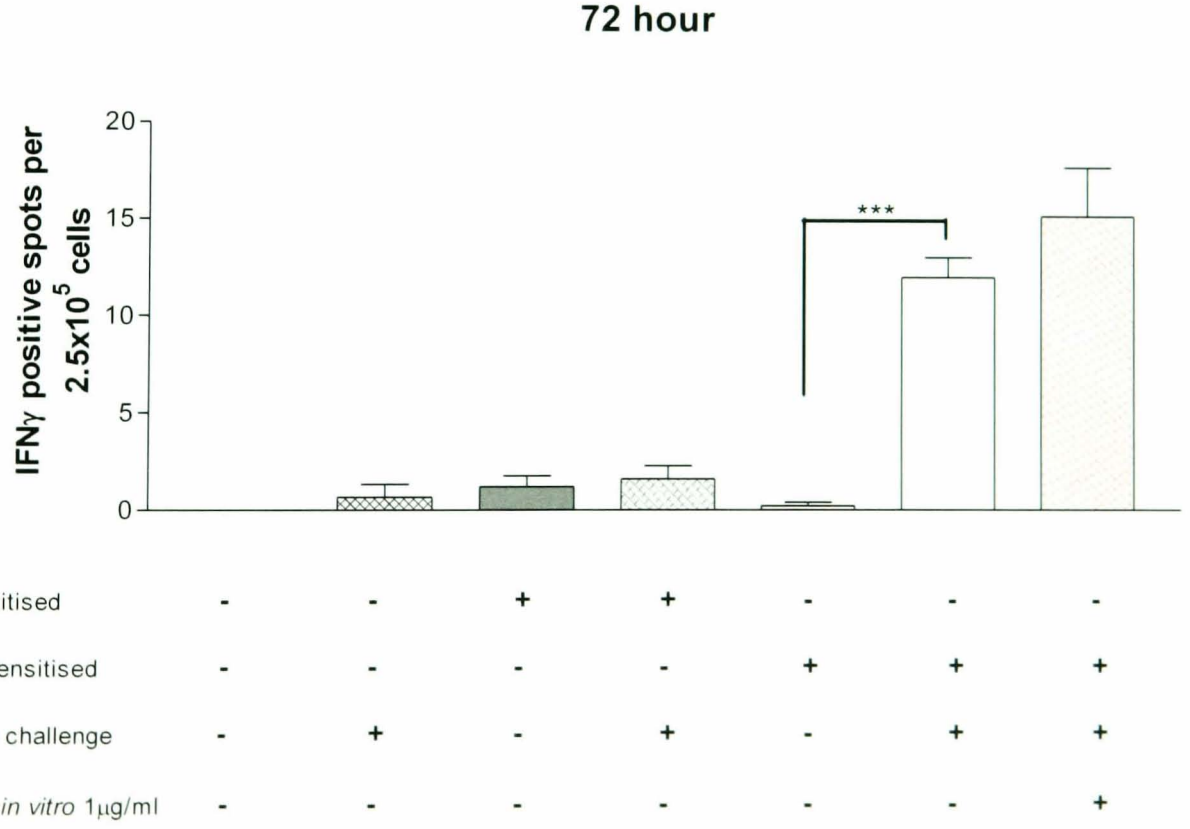
b.


72 hour
IL-17 positive spots per 2.5x10^5 cells
70
60
50
40
30
20
10
0
***
CFA sensitised - - + + - - - - -
CII/CFA sensitised - - - - + + + + +
CII in vitro challenge - + - + - + + + +
Anti-IFNγ in vitro 1μg/ml - - - - - - + - -
Anti-IFNγ in vitro 10μg/ml - - - - - - - + -
Anti-IFNγ in vitro 100μg/ml - - - - - - - - +

c.


24 hour
IFNγ positive spots per 2.5x10^5 cells
15
10
5
0
**
*
CFA sensitised - - + + - - - - -
CII/CFA sensitised - - - - + + + + +
CII in vitro challenge - + - + - + + + +
Anti-IL-17 in vitro 1μg/ml - - - - - - + - -
Anti-IL-17 in vitro 10μg/ml - - - - - - - + -
Anti-IL-17 in vitro 100μg/ml - - - - - - - - +

d.


72 hour
IFNγ positive spots per 2.5x10^5 cells
20
15
10
5
0
***
CFA sensitised - - + + - - -
CII/CFA sensitised - - - - + + +
CII in vitro challenge - + - + - + +
Anti-IL-17 in vitro 1μg/ml - - - - - - +

e.

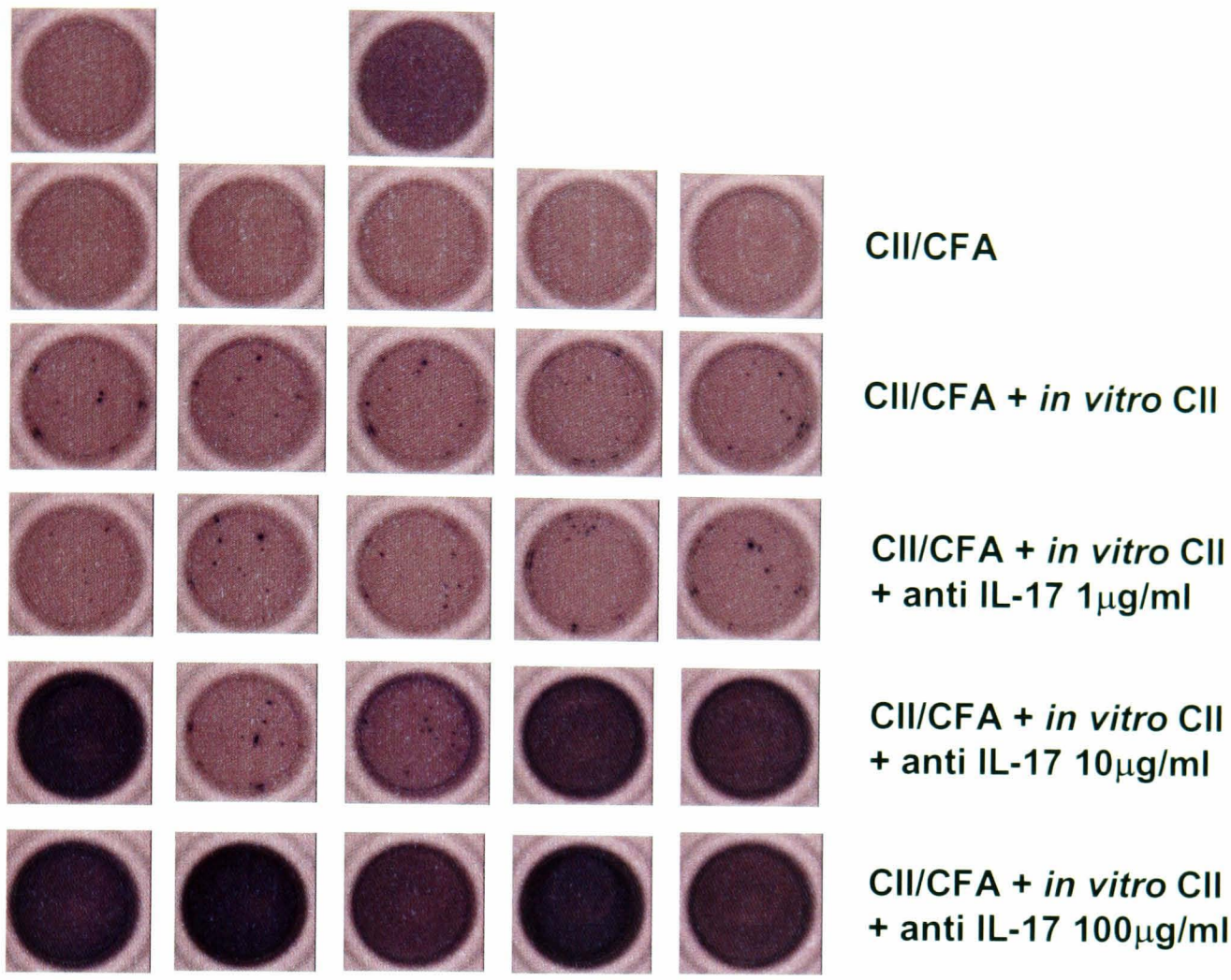


Figure 7.3 The effect of anti-IL-17 and anti-IFNγ on IFNγ and IL-17 positive spots respectively in the collagen II stimulated $CD4^+$ T cell assay.

$CD4^+$ T cells were prepared from inguinal lymph nodes of normal male DBA/1 mice or from mice 14 days after sensitisation with either complete Freund's adjuvant (CFA) or collagen II (CII) in CFA. Cells were cultured in 96 well IL-17 (a & b) and IFNγ (c, d & e) ELISpot plates at 2.5 x 10^5 $CD4^+$ T cells together with 1 x 10^5 mitomycin C treated antigen presenting cells obtained from normal spleens. Some wells were stimulated with 50μg/ml denatured CII. Anti-IFNγ and anti-IL-17 at 1, 10 and 100μg/ml were added to some wells in the IL-17 and IFNγ ELISpot plates respectively. Cells were cultured for 24 or 72 hours and assayed for IL-17 or IFNγ positive spots following the ELISpot protocol supplied with the kit. The numbers of IL-17 and IFNγ positive spots were counted in individual wells on an automated ELISpot reader. Addition of 10 and 100μg/ml anti-IL-17 to the IFNγ ELISpot plate resulted in wells that were too dark to count individual spots (e). Lymph nodes were

pooled from n = 5-10 mice per group and cells were plated out in replicates of 5. Data presented as mean ± s.e.m, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ statistically significant from indicated control.

7.3.3 Effect of recombinant mouse IL-17 (rmIL-17), IFNγ (rmIFNγ) and IL-4 (rmIL-4) in the CII stimulated CD4$^+$ T cell assay

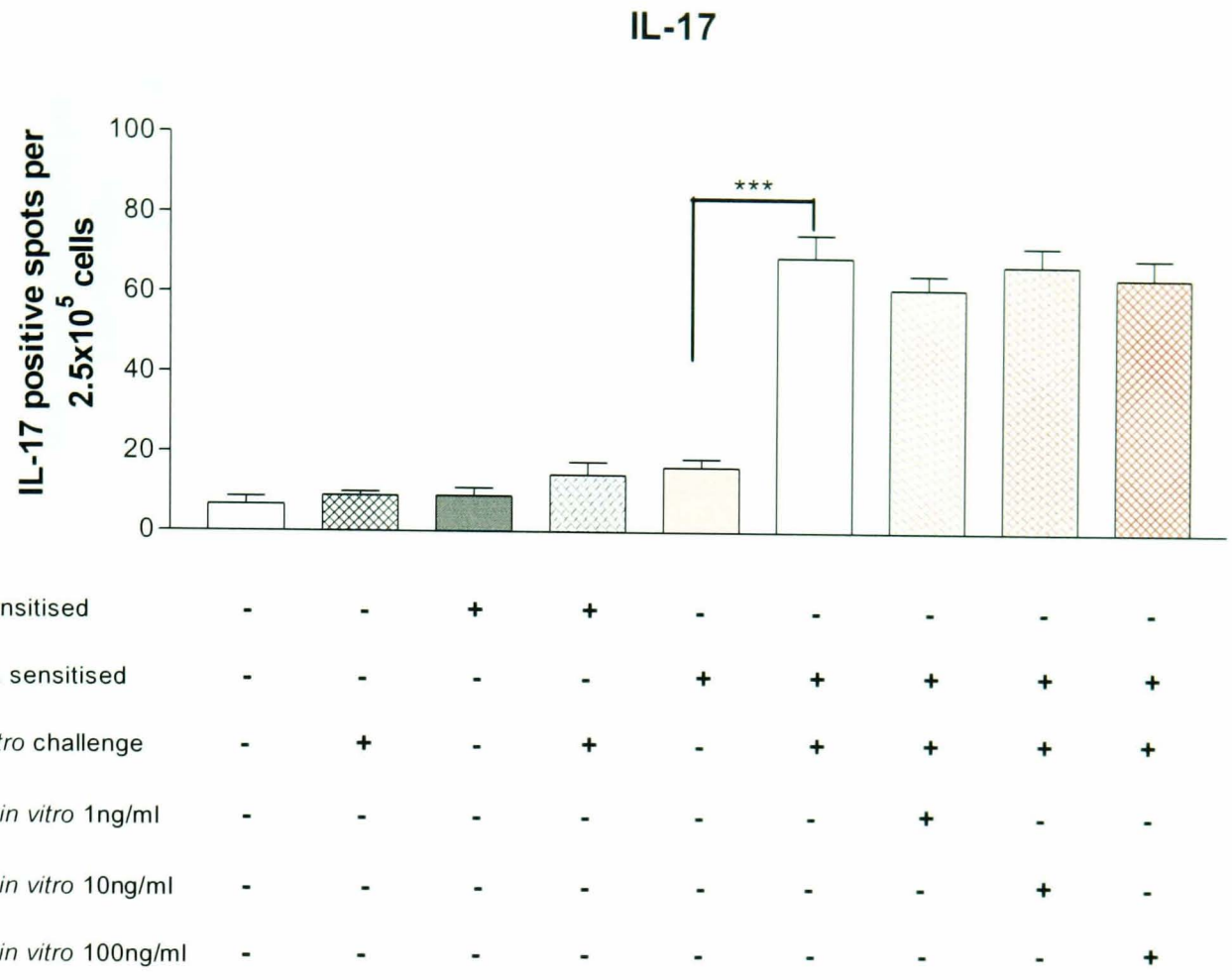
The relationship between IL-17, IFNγ and IL-4 in the CII stimulated CD4$^+$ T cell assay was investigated using recombinant mouse cytokines. Cell cultures were set up in cytokine specific ELISpot plates and cultured for 72 hours. The effect of rmIFNγ and rmIL-4 on CII stimulated IL-17 positive spots (Figure 7.4a and b), rmIL-17 and rmIL-4 on CII stimulated IFNγ positive spots (Figure 7.4c and d) and rmIL-17 and rmIFNγ on CII stimulated IL-4 positive spots (Figure 7.4e and f) was assessed.

Consistent with previous data, cells isolated from normal mice and CFA sensitised mice produced a low number of IL-17 and IFNγ positive spots and these cells did not respond to *in vitro* CII stimulation. In line with control cell responses, cells isolated from CII/CFA sensitised mice produced 16.40 ± 2.14 IL-17 positive spots and 1.20 ± 0.58 IFNγ positive spots. When these cells were stimulated with CII there was a statistically significant increase in the number of IL-17 positive spots which rose to 69.20 ± 5.70 (Figure 7.4a and b). The addition of rmIFNγ had no effect on the number of CII stimulated IL-17 positive spots detected in culture (Figure 7.4a). Adding rmIL-4 at 1 and 100ng/ml also had no effect on the frequency of CII stimulated IL-17 positive spots (Figure 7.4b). However, 10ng/ml rmIL-4 caused a statistically significant increase in the number of CII stimulated IL-17 positive spots (106.00 ± 7.44). CII stimulation of cells isolated from CII/CFA sensitised mice caused an increase in the number of IFNγ positive spots (6.60 ± 0.93 positive spots), however this was not statistically significant (Figure 7.4c and d). In the presence of rmIL-17 there was no statistically significant change in the number of CII stimulated IFNγ positive spots (Figure 7.4c). The addition of rmIL-4 at 1, 10 and 100ng mL

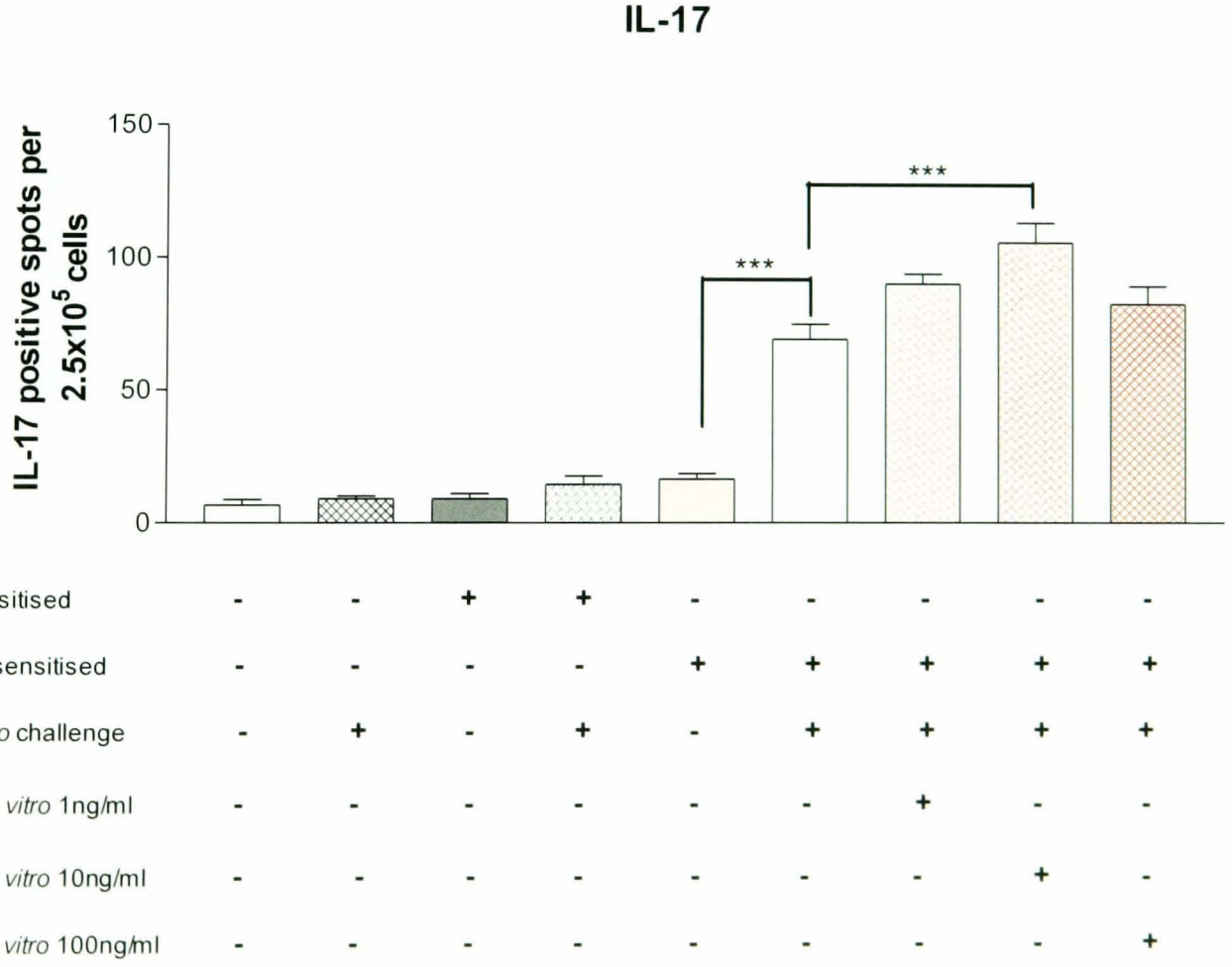
caused an increase in the number of CII stimulated IFNγ positive spots (13.00 ± 1.73, 30.20 ± 2.75 and to 30.80 ± 6.99 positive spots respectively). This increase was statistically significant at 10 and 100ng/mL of rmIL-4 (Figure 7.4d).

The frequency of IL-4 positive spots in the CII stimulated $CD4^+$ T cell assay was also assessed in this study (Figure 7.4e and f). In these assays only a few IL-4 positive spots were detected in control cell cultures and the addition of CII to these cells had no effect. Cells isolated from CII/CFA sensitised mice produced control levels of IL-4 positive spots (4.20 ± 0.92 positive spots) and this was not increased by CII stimulation (4.80 ± 1.24 positive spots). Culturing these cells with rmIL-17 and rmIFNγ had no effect on the number of IL-4 positive spots (Figure 7.4e and f).

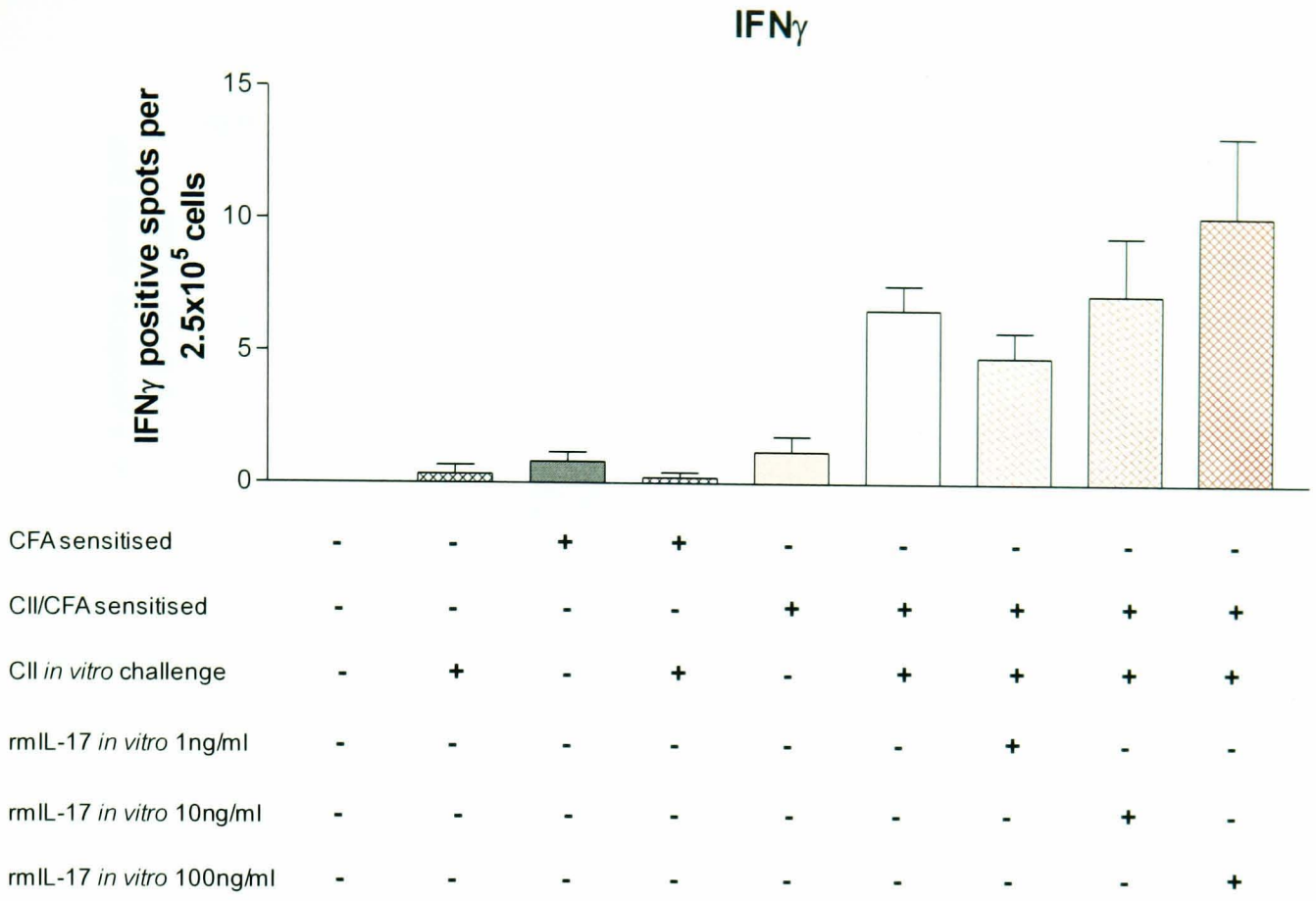
a.


IL-17
IL-17 positive spots per 2.5x10^5 cells
100
80
60
40
20
0
***
CFA sensitised - - + + - - - - -
CII/CFA sensitised - - - - + + + + +
CII in vitro challenge - + - + - + + + +
rmIFNγ in vitro 1ng/ml - - - - - - + - -
rmIFNγ in vitro 10ng/ml - - - - - - - + -
rmIFNγ in vitro 100ng/ml - - - - - - - - +

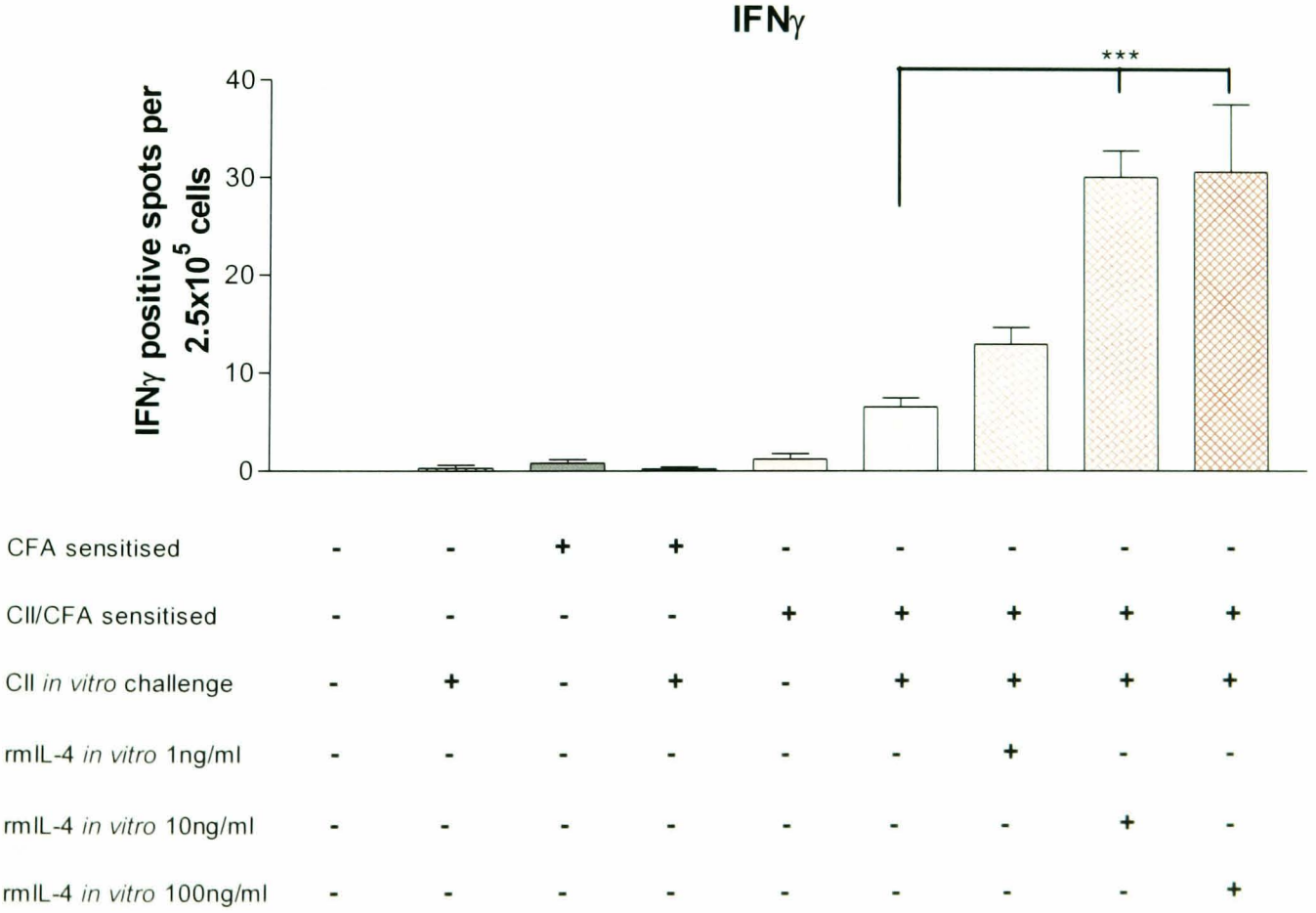
b.


IL-17
IL-17 positive spots per 2.5x10^5 cells
150
100
50
0
***
***
CFA sensitised - - + + - - - - -
CII/CFA sensitised - - - - + + + + +
CII in vitro challenge - + - + - + + + +
rmIL-4 in vitro 1ng/ml - - - - - - + - -
rmIL-4 in vitro 10ng/ml - - - - - - - + -
rmIL-4 in vitro 100ng/ml - - - - - - - - +

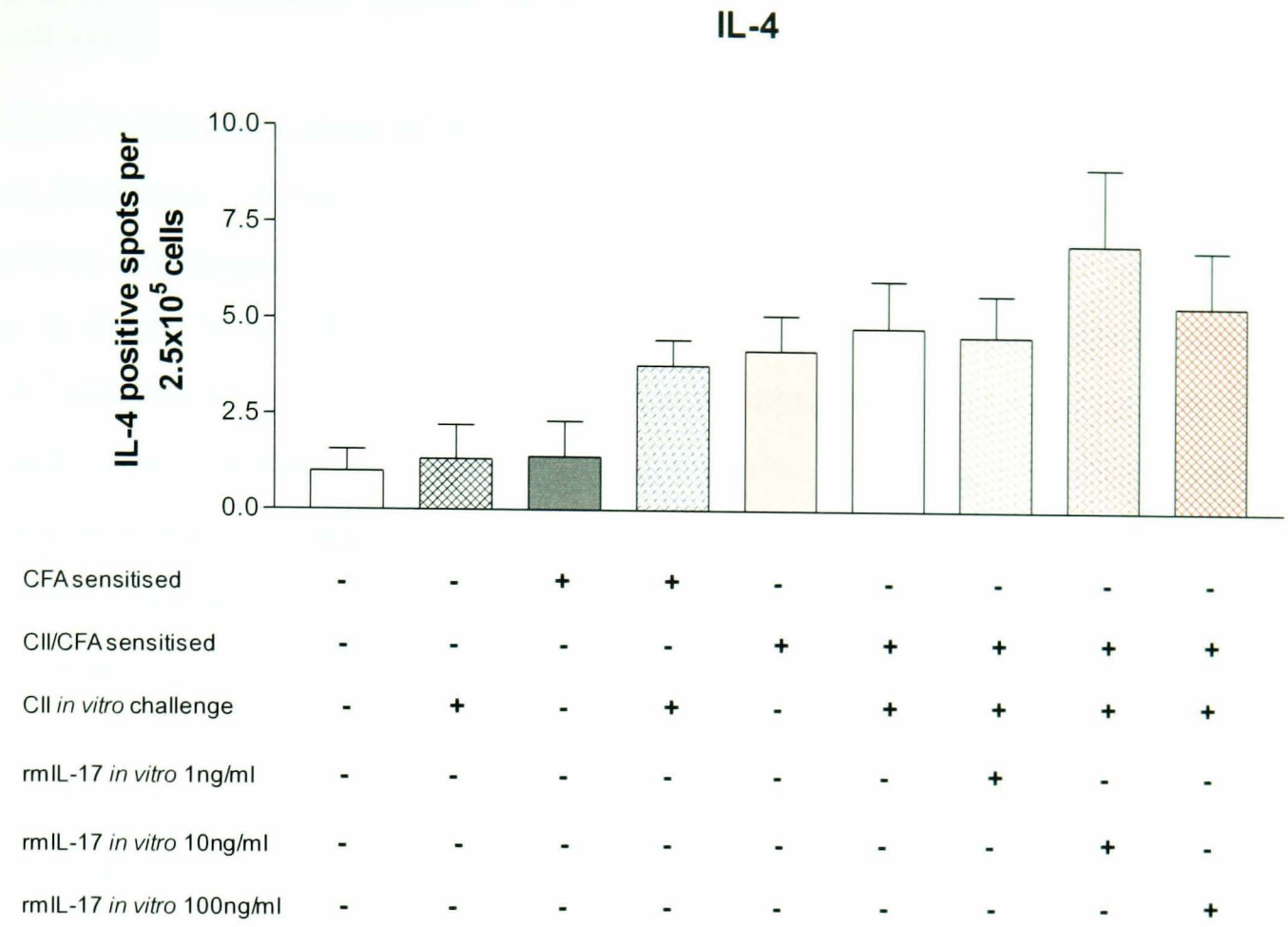
c.


IFNγ
IFNγ positive spots per 2.5x10^5 cells
15
10
5
0
CFA sensitised - - + + - - - - -
CII/CFA sensitised - - - - + + + + +
CII in vitro challenge - + - + - + + + +
rmIL-17 in vitro 1ng/ml - - - - - - + - -
rmIL-17 in vitro 10ng/ml - - - - - - - + -
rmIL-17 in vitro 100ng/ml - - - - - - - - +

d.


IFNγ
***
IFNγ positive spots per 2.5x10^5 cells
40
30
20
10
0
CFA sensitised - - + + - - - - -
CII/CFA sensitised - - - - + + + + +
CII in vitro challenge - + - + - + + + +
rmIL-4 in vitro 1ng/ml - - - - - - + - -
rmIL-4 in vitro 10ng/ml - - - - - - - + -
rmIL-4 in vitro 100ng/ml - - - - - - - - +

e.


IL-4
IL-4 positive spots per 2.5x10^5 cells
10.0
7.5
5.0
2.5
0.0
CFA sensitised - - + + - - - - -
CII/CFA sensitised - - - - + + + + +
CII in vitro challenge - + - + - + + + +
rmIL-17 in vitro 1ng/ml - - - - - - + - -
rmIL-17 in vitro 10ng/ml - - - - - - - + -
rmIL-17 in vitro 100ng/ml - - - - - - - - +

f.

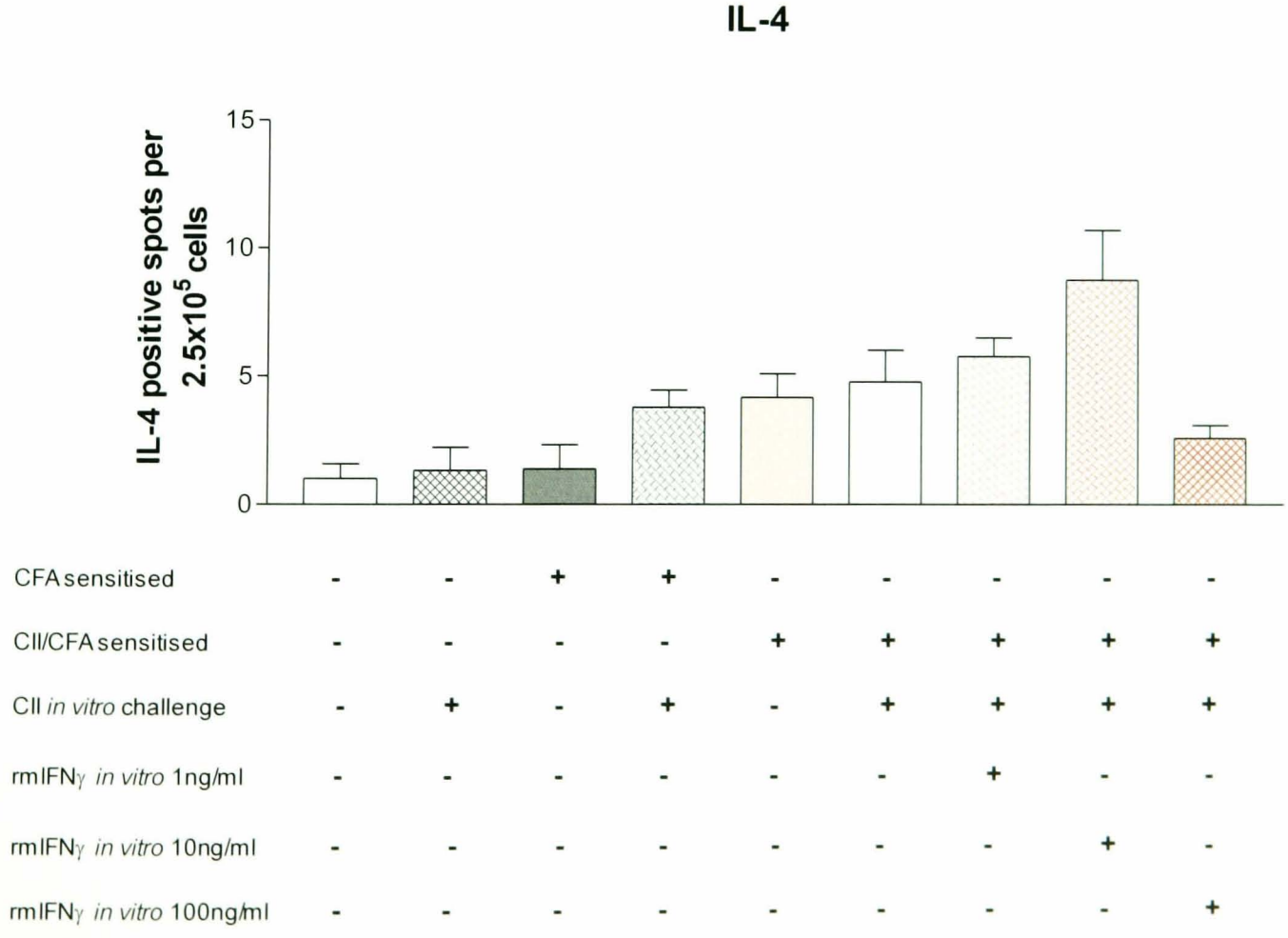

IL-4
IL-4 positive spots per 2.5x10^5 cells
15
10
5
0
CFA sensitised - - + + - - - - -
CII/CFA sensitised - - - - + + + + +
CII in vitro challenge - + - + - + + + +
rmIFNγ in vitro 1ng/ml - - - - - - + - -
rmIFNγ in vitro 10ng/ml - - - - - - - + -
rmIFNγ in vitro 100ng/ml - - - - - - - - +

Figure 7.4 The effect of recombinant mouse IL-17, IFNγ and IL-4 on the frequency of cytokine specific positive spots in the collagen II stimulated $CD4^+$ T cell assay.

$CD4^+$ T cells were prepared from inguinal lymph nodes of normal male DBA/1 mice or from mice 14 days after sensitisation with either complete Freund's adjuvant (CFA) or collagen II (CII) in CFA. Cells were cultured in 96 well IL-17 (a & b), IFNγ (c & d) and IL-4 (e & f) ELISpot plates at 2.5 x 10^5 $CD4^+$ T cells together with 1 x 10^5 mitomycin C treated antigen presenting cells obtained from normal spleens. Some wells were stimulated with 50μg/ml denatured CII. The effect of rmIL-17, rmIFNγ and rmIL-4 at 1, 10 and 100ng/ml was assessed on the frequency of cytokine specific spots in some wells. Cells were cultured for 72 hours and plates assayed for the presence of IL-17, IFNγ or IL-4 positive spots following the ELISpot protocol supplied with the kit. IL-17, IFNγ and IL-4 positive spots were counted in individual wells on an automated ELISpot reader. Lymph nodes were pooled from n = 5-10 mice per group and cells were plated out in replicates of 5. Data presented as mean ± s.e.m, *** $p<0.001$ statistically significant from indicated control.

7.3.4 Effect of in vivo anti-IL-17 and anti-IL-6 treatment on IL-17 production in the CD4$^+$ T cell assay

Samples from a CII stimulated CD4$^+$ T cell thymidine incorporation assay (Chapter 6 Figure 6.5) were used to address the question of whether *in vivo* treatment of CII/CFA sensitised mice with anti-IL-17 or anti-IL-6 would inhibit *ex vivo* IL-17 production.

IL-17 levels in cell culture supernatants from this study were analysed and results are shown in Figure 7.5. Control cells produced low levels of IL-17 and there was no statistically significant difference seen when these cells were stimulated with CII *in vitro*. Cells isolated from CII/CFA sensitised mice produced a similar amount of IL-17 as compared to controls (86.79 ± 36.47 pg/ml). Consistent with previous work these cells produced a statistically significant increase in IL-17 when stimulated with CII (2315.00 ± 564.00 pg/ml). Cells isolated from mice treated with anti-IL-17, cultured in the absence of CII, produced IL-17 at a similar level as non-stimulated cells from CII/CFA sensitised mice (132.80 ± 64.85 pg/ml). When cultured with CII these cells showed a reduction in IL-17 production as compared to CII stimulated cells from CII/CFA sensitised mice (1104.00 ± 765.40 pg/ml), however this was not statistically significant.

Strikingly, cells isolated from anti-IL-6 treated mice showed a statistically significant abrogation in CII stimulated IL-17 production (90.47 ± 76.35 pg/ml) as compared to cells from CII/CFA sensitised mice. In addition, non-stimulated cells from anti-IL-6 treated mice had no detectable levels of IL-17.

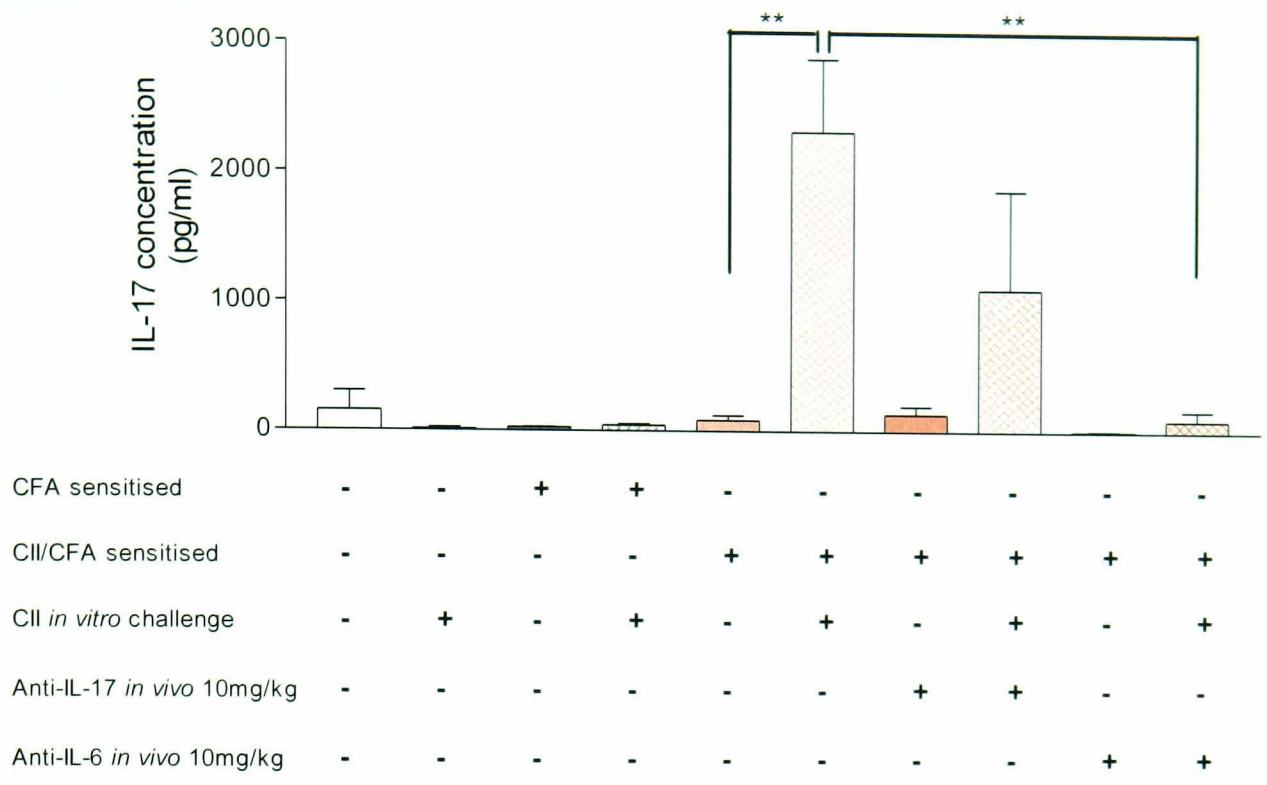


Figure 7.5 Effect of *in vivo* anti-IL-17 and anti-IL-6 on IL-17 production in the collagen II stimulated $CD4^+$ T cell assay.

$CD4^+$ T cells were prepared from inguinal lymph nodes of normal male DBA/1 mice or from mice 14 days after sensitisation with either complete Freund's adjuvant (CFA) or collagen II (CII) in CFA. Cells were plated out at 2.5 x 10^5 $CD4^+$ T cells together with 1 x 10^6 mitomycin C treated antigen presenting cells obtained from normal spleens. Some wells were stimulated with 50μg/ml denatured CII. In this experiment some animals were dosed with anti-mouse IL-17 or anti-mouse IL-6 antibodies at 10mg/kg s.c. once a week from one day prior to sensitisation. Cell supernatants were removed at 72 hours of culture and mouse IL-17 levels assessed by ELISA (R&D Systems). Lymph nodes were pooled from n = 5-10 mice per group and cells were plated out in replicates of 5. Data presented as mean ± s.e.m, ** $p<0.01$ statistically significant from indicated control.

7.4 Discussion

Table 7.1 and 7.2 summarise the thymidine incorporation, cytokine production and ELISpot data from the *ex vivo* CII stimulated $CD4^+$ T cell assays.

	$CD4^+$ T cell thymidine incorporation	**Cytokine levels**		
		IL-4	**IL-17**	**IFNγ**
CII/CFA controls	+	#	+	+
In vivo anti-IL-17	-		#	
In vivo anti-IL-6	-		-	

Table 7.1 Summary of thymidine incorporation, cytokine levels and drug effects in the *ex vivo* CII stimulated $CD4^+$ T cell assay.

	ELISpot		
	IL-4	**IL-17**	**IFNγ**
CII/CFA controls	#	+	+
In vitro anti-IL-17			+
In vitro rmIL-17	#		#
In vitro anti-IFNγ		#	
In vitro rmIFNγ	#	#	
In vitro rmIL-4		+	+

Table 7. 2 Summary of *in vitro* drug and recombinant mouse cytokine effects on *ex vivo* CII stimulated IL-4, IL-17 and IFNγ positive spots in the ELISpot assay.

Note: *+ represents a statistically significant increase and - represents a statistically significant decrease as compared to relevant control.*

 # represents no statistically significant change from relevant control.

Refer to Chapter 6 for $CD4^+$ T cell thymidine incorporation data.

In this chapter the role of IL-17 in CII immunity and in particular its relationship with IFNγ and IL-4 was investigated. It is clear from the data that $CD4^+$ T cells isolated from CII/CFA sensitised mice release IL-17 and IFNγ but not IL-4 in response to CII (Table 7.1).

In addition, IL-17 production from these cells was evident 24 hours post-CII stimulation, whereas IFNγ production was not seen until 48 hours. Furthermore, the amount of IL-17 detected at 72 hours in these cultures was 11.7 fold greater than IFNγ. The relative speed and magnitude at which IL-17 was produced compared to IFNγ suggests that IL-17 may be the dominant cytokine in these cultures and therefore infers an important role for Th17 cells in CII immunity.

To investigate the frequency of IFNγ, IL-4 and IL-17 secreting $CD4^+$ T cells in this assay, something that cannot be determined by measuring cytokine levels, ELISpot assays were set up. Consistent with the analysis of cytokine levels, cells isolated from CII/CFA sensitised mice showed an increase in the number of IL-17 and IFNγ positive spots in response to CII stimulation (Table 7.2). In addition, there were more IL-17 positive spots than IFNγ positive spots. The increase in IL-17 positive spots was statistically significant at 24 and 72 hours, whereas the increase in IFNγ positive spots only reached statistical significance at 72 hours. There was no increase in IL-4 positive spots in response to CII stimulation.

Taken together, these data strongly suggest that Th17 cells from sensitised mice are preferentially activated by CII and predominate over Th1 cells, while Th2 cells do not appear to have a role in the cellular arm of CII immunity 14 days post-CII sensitisation. There was also an increase in the number of CII stimulated IL-17 and IFNγ positive spots over time, which suggests Th17 and Th1 cells may be proliferating.

From the total number of $CD4^+$ T cells plated out in the ELISpot assay it would appear that only 1 in 11,905 (0.008%) secrete IL-17 and only 1 in 21,186 (0.005%) secrete IFNγ in response to CII stimulation at 72 hours of culture. This suggests that the population of Th17 and Th1 cells involved in CII immunity is very small.

To assess the relationship that may exist between IL-17 and IFNγ in CII immunity, anti-IL-17 and anti-IFNγ were added to ELISpot cell cultures. The addition of anti-IFNγ *in vitro* had no effect on the number of IL-17 positive spots either at 24 or 72 hours of culture, suggesting that IFNγ secreting Th1 cells are not capable of regulating the frequency of IL-17 secreting Th17 cells. However, the number of IFNγ positive spots in culture is low so the amount of IFNγ present may not be sufficient to affect Th17 cells, therefore blocking it has no effect. The addition of anti-IL-17 *in vitro* caused a concentration dependent increase in the number of IFNγ positive spots at 24 hours of culture. This suggests that IL-17 secreting Th17 cells may be involved in regulating the frequency of Th1 cells in these cultures and are capable of suppressing their development, something that has not previously been reported.

The relationship between these T cell subsets was investigated further in ELISpot assays using recombinant mouse (rm) IL-17 and rmIFNγ. In addition, the potential role that Th2 cells may have in these assays was investigated using rmIL-4. The *in vitro* addition of rmIFNγ had no effect on the number of CII stimulated IL-17 positive spots in culture, suggesting again that Th1 cells are not capable of regulating Th17 cells. In general, the presence of rmIL-4 had no effect on the number of IL-17 positive spots. However, 10ng/mL rmIL-4 did cause an increase in the number of IL-17 positive spots. It would therefore appear that, at certain concentrations, IL-4 may

be capable of regulating Th17 cells. However, endogenous IL-4 is extremely low and not CII specific in these cultures and is therefore not likely to influence the CII stimulated Th17 cell response. These data suggest that Th1 and Th2 cells are not capable of regulating committed CII specific Th17 cells, something that has not been reported before. In support of this, Harrington *et al.* (2005) reported similar findings using *in vitro* derived Th17 cells.

ELISpot assays have been designed to analyse the number of positive spots being formed but not the quantity of cytokine being produced, thus it is possible that Th1 and Th2 cells may still be able to alter IL-17 production. To address this possible effect, ELISpot assays should be run in conjunction with quantitative ELISA assays.

The increase in IFNγ positive spots seen when anti-IL-17 was added to cultures suggested that IL-17 may suppress Th1 cells and it was therefore anticipated that rmIL-17 would inhibit the number of IFNγ positive spots. However, the addition of rmIL-17 had no effect on the number of IFNγ positive spots. These assays have been developed to assess IL-17 positive spots with the number of IFNγ positive spots being minimal. It may therefore be difficult to detect a suppressive effect with exogenous IL-17 on such a low number of IFNγ secreting Th1 cells. Furthermore, the low number of IFNγ secreting Th1 cells detected in these cultures suggests that endogenous IL-17 may be efficiently suppressing these cells, therefore the addition of exogenous IL-17 has no additional effect. Hence these data cannot be used to discount a potential role for IL-17 in the regulation of IFNγ secreting Th1 cells. Further assays, optimised for IFNγ positive spots, are needed to assess the possible inhibitory effect of rmIL-17.

From the data presented so far it would appear that Th2 cells do not play a role in regulating cellular immunity to CII. It was therefore surprising that the addition of rmIL-4 caused an increase in the number of IFNγ positive spots. It is possible that in these cultures exogenous IL-4 represents an inappropriate Th2 cell response and in an attempt to suppress this, CII specific Th1 cells respond by proliferating and secreting IFNγ.

The addition of rmIL-17 and rmIFNγ to ELISpot cell cultures had no effect on the number of IL-4 positive spots which suggests that these cytokines do not regulate Th2 cells. However, the low frequency and lack of CII specificity of IL-4 secreting Th2 cells in these cultures makes it difficult to assess any inhibitory effects that IL-17 or IFNγ may have on Th2 cells.

In Chapter 6 it was demonstrated that *in vivo* treatment with anti-IL-17 and anti-IL-6 caused a statistically significant inhibition in CII stimulated $CD4^+$ T cell proliferation as compared to cells from non-treated CII/CFA sensitised mice. In this chapter IL-17 levels in the supernatants from these cell cultures were assessed. It was shown that *in vivo* treatment with anti-IL-17 caused a reduction in CII stimulated IL-17 production. However, this effect was not statistically significant suggesting that anti-IL-17 is not capable of preventing the formation of CII specific IL-17 secreting Th17 cells completely. Anti-IL-6 treatment caused a complete suppression in *ex vivo* CII stimulated IL-17 production from $CD4^+$ T cells. This finding suggests that anti-IL-6 may inhibit the formation of CII specific Th17 cells and therefore implies a role for IL-6 in their generation. In support of this, Veldhoen *et al.* (2006) demonstrated that IL-6 is important in Th17 cell differentiation from naïve $CD4^+$ T cells. The data

presented in the current study is the first to show that blocking IL-6 *in vivo* inhibits *ex vivo* IL-17 production in a CII immune response. The inhibition of Th17 cell formation may therefore represent a more novel mechanism of action by which anti-IL-6 exerts its anti-arthritic effect in CIA.

It is clear from the work conducted in this chapter that IL-17 secreting Th17 cells are important in CII immunity. It would appear that these cells are first to respond to re-stimulation with CII and produce greater quantities of IL-17 relative to the amount of IFNγ produced by Th1 cells, suggesting they dominate the immune response. The data has also indicated that Th1 and Th2 cells may not be capable of regulating CII specific Th17 cells and that, in fact, it is Th17 cells that may regulate Th1 cells in CII stimulated $CD4^+$ T cell cultures.

Chapter eight

Discussion

8.1 Discussion

Table 8.1 summarises drug effects on CIA and pre-arthritic readouts of CII immunity as assessed in this thesis.

Drug	**CIA**	**Hypersensitivity**		**Anti-CII antibodies**	**$CD4^+$ T cell proliferation**	
		6h	**24h**		***In vivo***	***In vitro***
Anti-$CD4^+$	+++	+++	+++	+++	**N/A**	**N/A**
Anti-$CD8^+$	-	-	-	-	**N/A**	**N/A**
Leflunomide	++	++	++	+++	+++	**N/A**
Anti-CD40L	+++	+++	+++	+++	+++	-
Anti-LFA-1	-	+++	+++	+++	+++	+++
Anti-TNFα	+++	-	-	++	-	-
Anti-IL-1β	+++	-	+	-	+++	++
Anti-IL-6	+++	-	+	++	+++	-
Anti-IL-17	+++	-	+	-	+++	-

Table 8.1 Summary of drug effects on CIA, CII hypersensitivity, anti-CII antibody production and CII stimulated $CD4^+$ T cell proliferation.

Note: *The data is semi quantitative where + represents a low, ++ represents a medium and +++ represents a high degree of suppression, all of which are statistically significant as compared to controls.*

- represents no significant change from control.

N/A represents not applicable.

Refer to Chapter 3 for CIA data, Chapter 5 for CII hypersensitivity and anti-CII IgG1/IgG2a data and Chapter 6 for CII stimulated $CD4^+$ T cell proliferation data.

8.2 $CD4^+$ T cell dependence of CIA and short-term readouts of CII immunity

In this thesis, anti-$CD4^+$ and anti-$CD8^+$ were used to characterise CIA and the short-term readouts of CII immunity. The literature has shown that CIA is $CD4^+$ T cell dependent (Kadowaki *et al.,* 1994, Chu and Londei. 1996, Ehinger *et al.*, 2001 and Taneja *et al.*, 2002) and data presented in this thesis confirms these findings. It has been demonstrated that $CD8^+$ T cells do not play a role in CIA, confirming work conducted by Ehinger *et al.* (2001). In addition, $CD8^+$ T cell depletion also had no effect on the short-term readouts, demonstrating that these cells are not involved in CII immunity in pre-arthritic mice. Depletion of $CD4^+$ T cells caused an inhibition in CII induced hypersensitivity and anti-CII Ab production, indicating that like CIA CII immunity in pre-arthritic mice is dependent on $CD4^+$ T cells.

8.3 Predicting anti-arthritic drug effects in CIA

The data presented in this thesis demonstrates that sensitisation of mice to CII causes humoral and cellular immune responses. Furthermore, these responses can be easily detected in pre-arthritic mice 14 days post-sensitisation to CII by assessing anti-CII Abs, CII induced hypersensitivity *in vivo* and CII stimulated $CD4^+$ T cell proliferation *ex vivo*. Part of the hypothesis to be tested was whether pre-arthritic readouts of CII immunity could be used to predict the anti-arthritic effects of drugs in the CIA model.

8.3.1 Drug effects in CIA and short-term readouts of CII immunity

In general, these models and assays which assess the immunomodulatory activity of a drug are indirectly predictive of anti-arthritic effects in CIA. However, it is clear from Table 8.1 that this is not always the case. For example, anti-LFA-1 failed to show a

statistically significant inhibition of CIA but did suppress CII induced hypersensitivity, anti-CII Ab production and CII stimulated $CD4^+$ T cell proliferation. indicating an inability of these models and assays to predict drug activity in CIA in this case. Other drugs were active in the CIA model but did not necessarily show suppressive activity in all the pre-arthritic readouts. For example, anti-TNFα did not inhibit CII hypersensitivity or $CD4^+$ T cell proliferation but did suppress anti-CII Ab production. In addition, anti-IL-1β and anti-IL-17 caused a statistically significant inhibition in CII induced hypersensitivity and $CD4^+$ T cell proliferation but had no effect on anti-CII Ab production. From these data it appears that the drugs investigated affect the immune response to CII in different ways, potentially providing a basis for discriminating between anti-arthritic drugs based on mechanism of action.

The drug effects observed in the CII hypersensitivity model at 24 hours post re-challenge generally mirror those seen in the CII stimulated $CD4^+$ T cell proliferation assay. This suggests that the hypersensitivity model at this time point is $CD4^+$ T cell dependent and is consistent with evidence obtained in Chapter 5 where anti-$CD4^+$ treatment abolished the hypersensitivity response. However, for some drugs there appears to be a discrepancy in the degree of suppression seen in the CII stimulated $CD4^+$ T cell proliferation assay and the 24 hour hypersensitivity response to CII. In particular it is evident that anti-IL-1β, anti-IL-6 and anti-IL-17 all had profound effects on CII stimulated $CD4^+$ T cell proliferation but only caused partial inhibition in the 24 hour hypersensitivity response.

What is not known in this model is the potential contribution that the immediate Ab mediated response seen at 6 hours may have on the delayed hypersensitivity response at 24 hours. It may be that some of the ear swelling at 24 hours in this model is

mediated through the immediate Ab response. If the Ab mediated response does contribute to ear swelling at 24 hours then this may explain the partial effect seen with anti-IL-1β, anti-IL-6 and anti-IL-17 as they had either no effect or a partial effect on anti-CII Ab production. In support of this, leflunomide, anti-LFA-1 and anti-CD40L not only suppressed $CD4^+$ T cell proliferation but also attenuated anti-CII Ab production resulting in a much greater inhibition of the 24 hour hypersensitivity response as compared to the anti-cytokine drugs.

To determine whether the Ab mediated response does contribute to 24 hour hypersensitivity, B cell deficient mice could be used. B cell deficient mice have dramatically reduced Ig levels but have normal T cell responses to CII (Svensson *et al.*, 1998). If B cell deficient mice showed a reduction in the 24 hour CII induced hypersensitivity response as compared to wild type mice this would suggest that the immediate Ab mediated response does contribute to ear swelling at 24 hours. Furthermore, if drugs that inhibit $CD4^+$ T cell proliferation but not anti-CII Ab production such as anti-IL-1β and anti-IL-17 were capable of greater suppression of the 24 hour response in B cell deficient mice as compared to wild type mice this would confirm a role for the Ab mediated response at 24 hours in normal mice.

Mice treated with anti-IL-1β, anti-IL-6 and anti-IL-17 showed a profound suppression of arthritis. However, the degree of suppression in the hypersensitivity response at 24 hours with these drugs, although statistically significant, was not profound. The hypersensitivity model measures the immune response against chick CII to which the mice have been sensitised. However, in the CIA model the endogenous release of CII in the joint of diseased mice is thought to perpetuate the immune response. It is likely that a mouse that has been sensitised to chick CII will

not mount such a strong immune response against mouse CII as it would against chick CII. Therefore, it may be easier to suppress the immune response in the CIA model than in the hypersensitivity model resulting in different drug effects between the models. It was shown in Chapter 4 that the hypersensitivity response was dependent on the amount of chick CII used for ear challenge with a lower dose showing a reduced response at both 6 and 24 hours. By reducing the amount of chick CII and hence the immune response in the hypersensitivity model it may be possible to mirror the effects of anti-IL-1β, anti-IL-6 and anti-IL-17 in the CIA model more closely.

Taken as a whole, the data suggests individual pre-arthritic readouts should not be used in isolation to predict anti-arthritic drug effects. However, the data does indicate that if these readouts are used in conjunction with one another they represent a strong basis for a pre-clinical screening cascade for anti-arthritic drugs, as set out in Figure 8.1. All drugs examined that were effective in CIA were effective in one or more of the short-term readouts. However, for some drugs such as anti-LFA-1 these short-term readouts may not always predict their anti-arthritic activity.

Many researchers use CII induced hypersensitivity, CII stimulated cell proliferation and anti-CII Ab isotype levels to try to understand how a drug is affecting CIA. However, not all readouts are used in the same studies and analysis is quite often done at the end of experiments. The use of these pre-arthritic models and assays as a screening cascade for predicting anti-arthritic drug effects is therefore a novel approach.

The short-term models developed in this thesis gave robust and reproducible readouts of CII immunity and used a reduced number of animals as compared to CIA. If these

readouts were to be used as a screening cascade they would therefore reduce the number of animals required for drug testing and the number that needed to be rendered arthritic. This has clear welfare advantages.

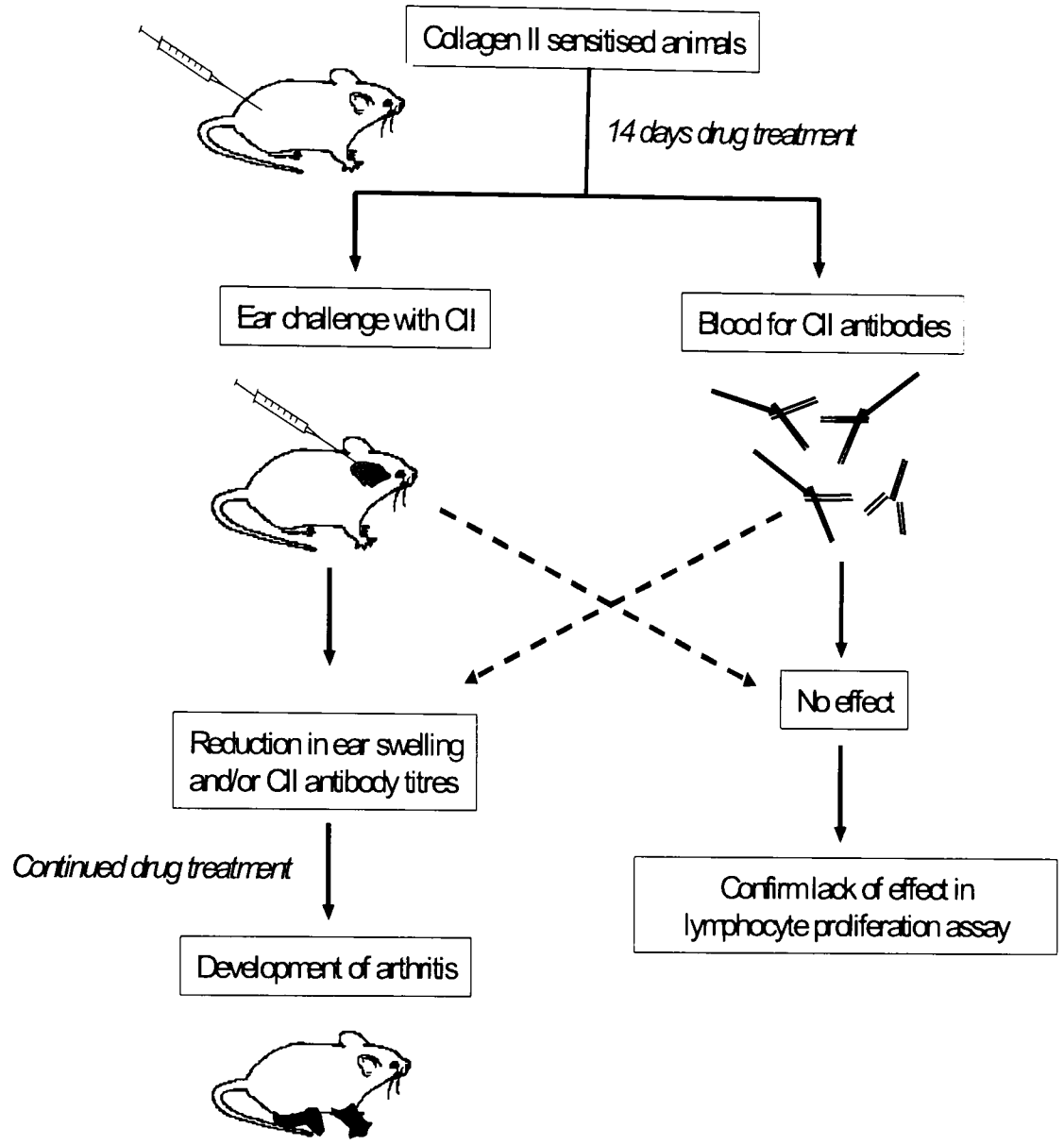


Figure 8.1 Proposed screening cascade for anti-arthritic drugs.

Drugs evaluated 14 days after CII sensitisation and reductions in ear swelling to CII challenge or circulating CII antibodies provide decision points as to whether to proceed to CIA. If no reduction is seen in these readouts then CII stimulated $CD4^+$ T cell proliferation is conducted to confirm lack of effect.

8.3.2 Prophylactic and therapeutic dosing regimes

The use of prophylactic dosing regimes, as opposed to therapeutic dosing regimes, in CIA and short-term models was deliberate. Events occurring immediately post-sensitisation with CII such as antigen uptake, proliferation and differentiation of effector cells provide many potential opportunities for immunomodulatory drugs. In a chronic disease such as RA these events are presumably ongoing and so the possibility exists that restricting the models to therapeutic dosing may not capture the immunomodulatory activities of some drugs.

In the clinic, administration of a depleting anti-$CD4^+$ mAb had moderate effects in RA patients (Tak *et al.*, 1995 and Choy *et al.*, 2000). Williams and Whyte. (1996) showed that depleting $CD4^+$ mAbs administered at the time of sensitisation suppressed CIA. However, when these Abs were given 3 weeks post-sensitisation they did not affect disease indicating that therapeutic dosing was not capable of predicting drug activity in the clinic in this instance. In addition, targeting the IL-6 receptor (IL-6R) with a mAb has been shown to be effective in RA (Nishimoto *et al.*, 2004), but therapeutic dosing of anti-IL-6R (Takagi *et al.*, 1998) and in our hands anti-IL-6 (historical data) had no effect on CIA whereas prophylactic dosing did. It would therefore appear that prophylactic dosing regimes in CIA and short-term models of CII immunity are more predictive of drug effects in the clinic than therapeutic dosing regimes. What cannot be ascertained using prophylactic dosing regimes in these models is how effective a drug will be clinically. It is likely that results from prophylactic studies will overestimate drug effects.

8.4 Drug mechanisms of action

The CIA model when used alone only really gives a yes or no answer in respect to the disease suppressing activity of a drug and does not enable the researcher to identify their mechanisms of action. The second part of the hypothesis to be tested was whether the pre-arthritic readouts could be used to differentiate between drugs, based on their mechanisms of action.

8.4.1 Mechanisms of action of leflunomide, anti-CD40L and anti-LFA-1

Leflunomide was used in this thesis to validate the CIA model and the short-term readouts of CII immunity. Leflunomide is a known anti-proliferative drug (Cherwinski *et al.*, 1995 and Chong *et al.*, 1993) and its anti-arthritic activity has been shown in experimental models of arthritis (Thoss *et al.*, 1996 and Schorlemmer and Schleyerbach. 1998). In the current study, the anti-arthritic activity of leflunomide was confirmed at a dose of 3mg/kg. However, this dose did not completely suppress CIA. In addition, CII stimulated $CD4^+$ T cell proliferation was inhibited at 3mg/kg but CII-induced hypersensitivity was not affected at this dose (historical data). Increasing the dose of leflunomide to 10mg/kg caused a statistically significant inhibition in CII-induced hypersensitivity and anti-CII Ab levels. These data again suggest that the hypersensitivity model may be more difficult to suppress than CIA. The effect of leflunomide on $CD4^+$ T cell proliferation, hypersensitivity and anti-CII Ab production demonstrated that these short-term readouts of CII immunity are capable of identifying drug mechanisms of action which were consistent with the known effects of leflunomide on lymphocytes.

Anti-CD40L and anti-LFA-1 were used to determine the importance of T cell – B cell and T cell – APC interactions respectively in CIA and CII immunity. In the current study, *in vivo* administration of anti-CD40L inhibited signs of arthritis in CIA and abrogated anti-CII Ab production in pre-arthritic mice. In support of this Durie *et al.* (1993) demonstrated that blocking CD40L-CD40 binding inhibited CIA with a corresponding reduction in anti-CII antibody levels. The role of T cell - B cell interactions in CIA was further highlighted by Tellander *et al.* (2000) who showed that administration of a stimulatory mAb against CD40 resulted in early onset and increased severity of disease. This was associated with increased anti-CII antibody production, suggesting the amount of CD40 stimulation during the immune response to CII may determine the severity of arthritis.

Anti-CD40L inhibited CII-induced hypersensitivity at 6 and 24 hours suggesting it may have an effect on T cells as well as Ab production. However, the influence of the immediate Ab mediated hypersensitivity response at 6 hours on 24 hour ear swelling is not known, therefore the inhibition of Ab production with anti-CD40L may contribute to the suppression of the 24 hour response. However, an inhibitory effect of *in vivo* anti-CD40L on T cells was confirmed in the *ex vivo* CII stimulated $CD4^+$ T cell proliferation assay. This suggests that CD40L is involved in the generation of CII specific $CD4^+$ T cells. In support of this Cayabyab *et al.* (1994) showed the importance of the CD40L-CD40 interaction in T cell proliferation using a cell line transfected with CD40 which augmented the proliferative response.

Further evidence suggests that CD40L itself can induce T cell proliferation in a T cell - T cell dependent manner (Fanslow *et al.*, 1994). In the current study, anti-CD40L failed to inhibit $CD4^+$ T cell proliferation when added *in vitro* suggesting CII specific T cell - T cell interactions are not essential in this assay. This lack of effect *in vitro*

also suggests that any B cells present in the naïve spleen preparation are not capable of stimulating T cell proliferation by acting as APCs. In support of this, Holmdahl *et al.* (2002) showed that naïve B cells did not activate CII specific T cell hybridomas as effectively as primed B cells from lymph nodes of CII sensitised mice therefore suggesting B cells act as APCs in disease. These data suggest that CII primed B cells may play a role in T cell proliferation and this may be through increased CD40 expression. Therefore, it appears that blocking CD40L *in vivo,* not only inhibits B cell Ab production, but also stops CD40 from stimulating T cell proliferation suggesting there is a reciprocal stimulatory pathway between T cells and B cells.

The profound effect of anti-LFA-1 in the pre-arthritic models and assays would suggest it is, potentially, a potent anti-arthritic drug. Anti-LFA-1 had an effect on humoral immunity as it attenuated the production of anti-CII isotypes and inhibited the 6 hour CII-induced hypersensitivity response. Blocking LFA-1 also attenuated the 24 hour CII-induced hypersensitivity response indicating it also has an effect on cellular immunity, a result supported by its inhibitory effect on CII stimulated $CD4^+$ T cell proliferation, both *in vivo* and *in vitro*. This confirms work by Dongworth *et al.* (1985) and Berzins *et al.* (1988) who showed that Abs directed against LFA-1 inhibit T cell proliferation. These data indicate that T cell – APC contact is essential in an immune response and suggest that LFA-1 is important in initial immunity to CII. In support of this, Hersmann *et al.* (1998) reported that LFA-1 is strongly expressed in the joint of mice in the acute phase of arthritis. However, the same study demonstrated that LFA-1 expression in the joint decreases in the chronic phase of disease. This suggests that LFA-1 may not be as important later on in CIA and work conducted in this thesis appears to confirm this as anti-LFA-1 treatment only caused a partial inhibition of arthritis.

Mikecz *et al.* (1994) showed that blocking LFA-1 and its ligand, ICAM-1, only gave a modest reduction in signs of arthritis. Whereas Kikimoto *et al.* (1992) reported that mAbs against LFA-1 and its ligand ICAM-1 caused a statistically significant suppression in CIA. However, even in this paper, the suppression was not profound. In contrast to the effect of anti-LFA-1 on anti-CII Abs in the current study Kikimoto demonstrated that blocking the interaction between LFA-1 and ICAM-1 did not affect anti-CII Ab production and concluded that anti-LFA-1 only affects cellular immunity. In the study by Kikimoto, anti-CII Ab production was assessed on Day 35 post-sensitisation as compared to Day 15 in this thesis. It is therefore possible that an earlier inhibitory effect of blocking LFA-1 activity on humoral immunity may have contributed to the attenuation of disease in their study. Taken together, these data indicate that anti-LFA-1 is not an effective anti-arthritic drug in the CIA model.

The bond between ICAMs and LFA-1 is not exclusive. For example, ICAM-3 and LFA-2 (CD2) expressed on T cells can also bind DC-SIGN, a recently identified lectin on DCs, and LFA-3 (CD58) on APCs respectively, suggesting Ag recognition can occur without LFA-1 involvement. Thus, cellular adhesion between T cells and APCs may primarily be dependent on LFA-1, but in its absence these other interactions can compensate and this is why there is a delay in arthritis in the CIA model but not an inhibition.

LFA-1 is also important in cell recruitment to sites of inflammation, again via ICAM binding, which may explain the inhibitory effect seen with anti-LFA-1 in the hypersensitivity model. However, another integrin, very late activation antigen 4 (VLA-4), is expressed on activated T cells and binds its ligand VCAM-1 which is up regulated on endothelium at sites of inflammation. This integrin is expressed later on in the inflammatory response, hence its name, and is able to recruit T cells. In a

chronic inflammatory environment, such as an arthritic joint. VLA-4 may be able to compensate for the lack of LFA-1 and recruit T cells, thus helping to render anti-LFA-1 treatment less effective. It is possible that VLA-4 is not expressed in the acute 24 hour hypersensitivity model so LFA-1 is the dominant integrin in respect to mediating cell accumulation.

It appears from the data that there may be a temporal difference in the underlying immune process between the short-term readouts and CIA. In terms of predicting anti-arthritic activity this may lead to either a false positive result, as in the case of anti-LFA-1, or perhaps a false negative result in the short-term models.

8.4.2 Mechanisms of action of anti-TNFα, anti-IL-1β and anti-IL-6

TNFα and IL-1β are well studied cytokines with a wide range of pro-inflammatory actions. It was evident from the short-term readouts of CII immunity that TNFα had little immunomodulatory activity as blocking it with anti-TNFα had no effect on cellular immunity. This was indicated by a failure to inhibit the 24 hour hypersensitivity response and CII stimulated $CD4^+$ T cell proliferation. This finding confirms work by Campbell *et al.* (2001) who showed that TNF deficient mice have normal T cell proliferation, in response to CII and Choy *et al.* (1999) who showed that anti-TNFα therapy has no effect on T cell activation in RA patients. In the pre-arthritic hypersensitivity model, anti-TNFα did reduce anti-CII IgG1 and IgG2a production, suggesting it may affect the humoral response to CII and that this could contribute to its anti-arthritic activity in the CIA model. This reduction in anti-CII Abs did not translate to an effect on 6 hour hypersensitivity suggesting there is still enough circulating anti-CII Ab present to elicit ear swelling. Williams *et al.* (1992) had previously shown anti-TNFα treatment inhibits CIA, but in contrast to the present

study showed no effect on anti-CII IgG production. In this paper, anti-CII IgG was assessed in the arthritic phase of disease and it may be that other compensatory mechanisms have had a chance to elevate Ig levels at this time point. Further evidence to support the role of TNFα in the humoral response comes from TNF deficient mice that have impaired Ig class switching (Campbell *et al.*, 2001) therefore suggesting the humoral response is compromised by a lack of TNFα. Also, a study by Kehrl *et al.* (1987) showed that activated B cells have prolonged DNA synthesis, increased TNFα binding sites and increased Ig secretion when stimulated with TNFα and IL-2. These data suggest that TNFα has a role to play in humoral immunity and that anti-TNFα may have some minor immunomodulatory activity.

TNFα has been shown to regulate the production of IL-1 (Brennan *et al.*, 1989 and Williams *et al.*, 2000) and IL-6 (Choy *et al.*, 1999). This indirect effect on IL-6 may explain the inhibition in anti-CII Ab production in the pre-arthritic model. However, if anti-TNFα treatment were having a profound effect on IL-1β and IL-6 production an effect on CII-induced hypersensitivity and $CD4^+$ T cell proliferation would be predicted, similar to that of blocking these cytokines directly. It may be that anti-TNFα inhibits at least in part IL-6, resulting in reduced anti-CII antibody production, but does not suppress IL-6 and IL-1β enough to inhibit cellular immunity.

The biological actions of TNFα are predominately pro-inflammatory therefore anti-TNFα is likely to be exerting the majority of its anti-arthritic activity by inhibiting inflammation. The models and assays in this thesis are not designed to detect drug effects on non-immune inflammation and this is one weakness in the use of these readouts as a screening cascade for novel anti-arthritic agents. A readout of non-immune inflammation such as neutrophil accumulation may be a useful addition to such a screening cascade.

Anti-IL-1β attenuated signs of arthritis in CIA indicating that IL-1β is a key cytokine in this model. In support of this IL-1 has been shown to accelerate signs of disease in CIA (Killar and Dunn. 1989 and Hom *et al.*, 1988). In the short-term readouts of CII immunity anti-IL-1β reduced ear swelling at 24 hours and inhibited CII stimulated $CD4^+$ T cell proliferation both *in vivo* and *in vitro*. However, anti-IL-1β failed to inhibit the humoral response, as indicated by no effect on 6 hour CII induced ear swelling or anti-CII Ab production. This suggests that anti-IL-1β may exert its anti-arthritic effect at least in part by modulating cellular immunity to CII. These data confirm results obtained in a CIA study using IL-1 deficient mice, which showed normal Ab but decreased T cell responses (Saijo *et al.*, 2002).

The *in vitro* effect of anti-IL-1β in the CII stimulated $CD4^+$ T cell proliferation assay suggests that IL-1β may have a direct effect on the cells in culture. This was not evident with any other anti-cytokine treatment. It has been shown that IL-1β stimulates the production of IL-2 and induces the expression of its receptor, IL-2R, (Hackett *et al.*, 1988) and can synergise with IL-6 to increase IL-2 production (Holsti and Raulet. 1989), with IL-2 being vital in T cell proliferation. Cyclosporin effectively inhibited CII stimulated $CD4^+$ T cell proliferation *in vitro* demonstrating that the assay is dependent on IL-2, as cyclosporin acts by disrupting signalling through the T cell receptor and inhibits IL-2 production. Blocking IL-1β *in vitro* may therefore inhibit the production of IL-2 from T cells and IL-2R expression on T cells, thus attenuating CII stimulated $CD4^+$ T cell proliferation.

Another explanation as to how anti-IL-1β may be inhibiting proliferation *in vitro* again comes from the study in IL-1 deficient mice (Saijo *et al.*, 2002). These authors showed that the absence of IL-1 causes a decrease in CD40L and OX40 (CD134) expression on T cells. The data in Chapter 6 indicates that CD40L does not play a

role in T cell proliferation *in vitro*, therefore ruling it out as a mechanism by which anti-IL-1β had its effect in this assay. However, the costimulatory molecule OX40 is a potential candidate for regulation by IL-1β *in vitro*. This theory is supported by Nakae *et al.* (2001) who showed that IL-1 enhances T cell priming through induction of OX40 on T cells. The relevance of OX40 - OX40L binding in CIA has been demonstrated by Yoshioka *et al.* (2000) using a mAb against OX40L which successfully inhibited arthritis. OX40 is also expressed on $CD4^+$ T cells in synovial fluid from RA patients (Giacomelli *et al.*, 2001), suggesting it is of clinical relevance. A way to ascertain if OX40 is involved *in vitro* in $CD4^+$ T cell proliferation would be to use an anti-OX40 mAb something that has not been undertaken in this thesis.

These data suggest the *in vitro* effect of anti-IL-1β on T cell proliferation may be due to blocking the action of IL-1β on OX40 and IL-2/IL-2R expression. These effects may also explain the potent *in vivo* activity of anti-IL-1β treatment on $CD4^+$ T cell proliferation.

The precise mechanisms by which anti-IL-1β is causing its anti-arthritic effect cannot be fully identified in these pre-arthritic readouts. Literature evidence suggests that, like anti-TNFα, anti-IL-1β is probably exerting many of its anti-arthritic effects by inhibiting inflammation.

Another pivotal cytokine in the immune response is IL-6, which again has pleotropic actions. Anti-IL-6 treatment caused an inhibition in anti-CII antibody production, CII-induced ear swelling at 24 hours and CII stimulated $CD4^+$ T cell proliferation. These data suggest that anti-IL-6 is an immunomodulatory agent capable of affecting both the humoral and cellular components of the immune system. With these effects on CII immunity it was not surprising that anti-IL-6 treatment caused a statistically

significant inhibition in CIA. These results demonstrate that IL-6 is another essential cytokine in the immune response to CII. In support of this Alonzi *et al.* (1998) showed that IL-6 deficient mice are protected against CIA with a corresponding decrease in anti-CII Ab levels. In these IL-6 deficient mice the anti-CII Ab response was not totally inhibited, a result similar to that described here. This suggests anti-CII Ab production is not totally dependent on IL-6. This may explain why anti-IL-6 failed to inhibit the 6 hour hypersensitivity time point as there is still enough circulating anti-CII Ab to cause a response. This reduction in anti-CII IgG1 and IgG2a Ab production may be due to blocking the direct effect of IL-6 on B cells, which has been reported to augment the generation of Ig secreting cells and secretion of Ig. IL-6 has also been demonstrated to enhance T cell dependent induction of B cell differentiation and this is thought to be due in part to IL-2 (Splawshi *et al.*, 1990). Antibodies against IL-6 and IL-2 have been shown to inhibit Ig production in $CD4^+$ T cell driven assays, supporting a role for IL-6 and IL-2 in B cell activation (Croft and Swain. 1991). Another study by Takagi *et al.* (1998) showed that blocking the IL-6R with a mAb also inhibited CIA when dosed at or just after sensitisation with CII and caused a decrease in the humoral response. In this study, a decrease in anti-CII IgG2a and IgG2b was detected, but not IgG1, suggesting there could be a shift towards a Th2 cell response that may be protective in CIA. However, anti-IL-6 treatment in the present study did inhibit IgG1 levels suggesting this is not the case 14 days post-sensitisation. Takagi *et al.* assessed levels of anti-CII Abs on Day 34 post-sensitisation suggesting that if a shift towards a Th2 response does occur, it takes longer than two weeks of treatment.

CII stimulated cell proliferation in the study by Takagi *et al.* was completely inhibited by anti-IL-6R mAb and supports findings reported here. It has also been shown that

IL-6 is capable of inducing proliferation of thymocytes and can synergise with IL-1 in this respect (Helle *et al.*, 1988), therefore playing a role in the development of thymic T cells. Blocking IL-6 could reduce the number of T cells in the circulation that are capable of seeing CII thereby preventing T cell priming and the initiation of the immune response. This would explain the profound effect seen with anti-IL-6 in the CII stimulated $CD4^+$ T cell proliferation assay. A lack of CII specific T cells would also reduce the amount of help available for stimulating the humoral response and could explain in part the reduction in anti-CII Abs.

IL-6 has been shown to promote T cell proliferation and increases the accumulation of stable IL-2 transcription in stimulated T cells, with IL-2 amplifying the proliferative response (Pankewycz *et al.*, 1990). IL-6 has also been reported to have synergistic effects with IL-1 in respect to these responses (Holsti and Raulet. 1989). Anti-IL-6 may therefore inhibit IL-2 production and attenuate CII stimulated $CD4^+$ T cell proliferation.

Anti-IL-6 had no effect when added *in vitro* in the CII stimulated proliferation assay. Pankewycz *et al.* (1990) used anti-CD3 stimulation of resting T cells whereas the cells in the *in vitro* CII assay here were stimulated *in vivo* with CII so are already CII specific. This could explain differences in findings between Pankewycz and the results presented here. It may be that prophylactic *in vivo* anti-IL-6 treatment inhibits the generation of CII specific T cells from naïve cells but cannot suppress CII specific $CD4^+$ T cells themselves. In support of this, Vink *et al.* (1990) showed that IL-6 was critical for initiating rather than maintaining T cell proliferation, as anti-IL-6 lost its inhibitory effect when added late in a T cell assay. This may explain why blockade of IL-6 signalling with an Ab against the IL-6R dosed on Day 7 post-sensitisation or later had no effect on CIA (Takagi *et al.*, 1998).

In Chapter 7, it was shown that *in vivo* treatment with anti-IL-6 inhibited IL-17 production in the *ex vivo* CII stimulated $CD4^+$ T cell assay. This suggests IL-6 is capable of regulating the production of IL-17 *in vivo*. IL-17 is another important cytokine involved in the immune response and has a number of actions such as the induction of TNFα, IL-1β and IL-6 which have important roles to play in inflammation and immunity and it has been shown in this thesis that blocking IL-17 inhibits CII immunity and CIA. It is possible that anti-IL-6 may exert part of its anti-arthritic effect by inhibiting IL-17 production. In support of this, Veldhoen *et al.* (2006) and Bettelli *et al.* (2006) recently showed that IL-6 in the presence of TGFβ supports the differentiation of naïve $CD4^+$ T cells into IL-17 producing T cells. Anti-IL-6 may therefore inhibit the formation of IL-17 producing cells *in vivo* thereby blocking IL-17 and its immunological actions. The use of naïve $CD4^+$ T cells in the papers by Veldhoen and Bettelli suggests this process may occur early on in the immune response to CII. This could again explain why blocking the IL-6R has little effect in CIA 7 days post-sensitisation as these IL-17 cells may have already differentiated. It would be interesting to see if IL-6 and TGFβ are capable of supporting further differentiation and activation of IL-17 producing cells in an already established immune response. The differentiation of IL-17 producing cells by IL-6 and TGFβ can be amplified with IL-1β and TNFα (Veldhoen *et al.*, 2006) suggesting another possible mechanism of action of anti-IL-1β and anti-TNFα therapy.

It has been shown in CIA, that regulatory T cells may have a protective role to play, as depletion of these cells results in exacerbation of disease (Morgan *et al.*, 2003) and TGFβ has been implicated in the production of $CD4^+CD25^+$forkhead box protein 3^+ ($Foxp3^+$) regulatory T cells (Treg cells). However, in the presence of IL-6 their

generation is completely inhibited (Bettelli *et al.*, 2006). Blocking IL-6 may therefore increase the formation of a Treg cell population and inhibit CII immunity.

In another model of arthritis, Wong *et al.* (2006) showed that $CD4^+$ lymphocytes from IL-6 deficient mice had reduced antigen-induced proliferation and produced less IL-17 than cells from wild type mice which is in support of the data in this thesis. It would have been interesting to see if the addition of anti-IL-6 *in vitro* in the CII stimulated $CD4^+$ T cell proliferation assay was able to inhibit IL-17 production. This could indicate whether IL-6 can support IL-17 producing cells in an established immune response. It has been shown that blocking IL-17 therapeutically in the CIA model, in our hands (historical data) and in the literature (Lubberts *et al.*, 2004), inhibits disease. However, the lack of effect seen with anti-IL-6R Ab when dosed later in disease suggests that anti-IL-6 may not inhibit IL-17 production.

It would appear IL-6 plays a vital role in CII immunity and is capable of inducing B and T cell responses as well as supporting the generation of IL-17 producing cells and may synergise with IL-1β and TNFα. Blocking IL-6 therefore inhibits CII immunity and CIA, suggesting that this pleotropic cytokine is an attractive target for therapeutic intervention. These pre-arthritic readouts have been able to identify some of the mechanisms of action of anti-IL-6 and the analysis of IL-17 in cell culture supernatants has suggested a novel mechanism of action. Routine cytokine analysis of cell culture supernatants may be of value when trying to understand drug effects in an immune response.

8.4.3 Mechanisms of action of anti-IL-17

In recent years the pro-inflammatory cytokine, IL-17, has been shown to be produced by T cells resident in synovial tissue of RA patients and appears to mediate inflammation (Chabaud *et al.*, 1999) and has therefore become a focus for possible therapeutic intervention. IL-17, like many of the other cytokines discussed, has a plethora of biological activities. Among these actions is its ability to synergise with IL-1β to enhance IL-6 production (Chabaud *et al.*, 1998) and with TNFα to enhance both IL-6 and IL-1β production from synoviocytes (Katz *et al.*, 2001). This suggests another mechanism of action for both anti-IL-1β and anti-TNFα as they can potentially inhibit this synergistic relationship with IL-17.

IL-17 has also been implicated in the destruction of cartilage and bone in arthritis, where it has been shown to induce matrix metalloproteinases (Jovanovic *et al.*, 1998 and Park *et al.*, 2005), osteoclastogenesis (Sato *et al.*, 2006), collagen destruction (Chabaud *et al.*, 2001) and expression of RANKL (Lubberts *et al.*, 2004).

Blockade of IL-17 with a mAb, in the current study, inhibited CIA. These data confirm research where IL-17 deficient mice (Nakae *et al.*, 2003) and other blocking strategies have been shown to attenuate arthritis (Lubberts *et al.*, 2001 and Lubberts *et al.*, 2004). Additionally, systemic over-expression of IL-17 by adenoviral vector has been shown to increase the severity of CIA (Lubberts *et al.*, 2001). These data indicate a key pathogenic role for IL-17 in CIA.

Assessment of anti-IL-17 in the pre-arthritic readouts showed a profound effect on CII immunity, as it inhibited CII stimulated $CD4^+$ T cell proliferation and CII-induced hypersensitivity in the ear at 24 hours. However, no effect was seen on anti-CII Ab levels or 6 hour hypersensitivity, suggesting anti-IL-17 exerts its effect preferentially on cellular immunity. In support of this, the work conducted by Nakae

et al. (2003) demonstrated that IL-17 deficient mice had a reduced T cell response to CII. In this study they also saw a reduced IgG2a response, which is in contrast to the current study. However, Nakae *et al.* took blood samples at termination of their study on Day 60, as compared to Day 15 in the present study. We too show an inhibition in anti-CII Abs at the end of a CIA study (historical data). Anti-IL-17 therefore appears to have an effect on the humoral response later on in disease, maybe through a delayed effect of inhibiting $CD4^+$ T cells.

Lubberts *et al.* (2001) showed that, although blocking IL-17 inhibited CIA, it had no effect on the level of anti-CII Abs or CII stimulated T cell proliferation and concluded that IL-17 had no effect on CII immunity. However, in this paper, the authors dosed an IL-17R:Fc fusion protein at boost or onset of disease symptoms. Data presented here indicates that IL-17 may play an important role initially in CII immunity but its immunomodulatory activity may be lost once the immune process has become established. The effects of anti-IL-17 dosed therapeutically in CIA suggest that, at this stage of disease, the effects of anti-IL-17 may be anti-inflammatory rather than immunomodulatory.

IL-17 has also been shown to be involved in the induction of IL-1β, TNFα and IL-6 (Jovanovic *et al.,* 1998) therefore blocking IL-17 may inhibit the production of these cytokines as well as their associated synergies and related immunomodulatory activity. It has been shown that anti-IL-1β and anti-IL-6 inhibit $CD4^+$ T cell proliferation when dosed *in vivo*. This suggests the effects of anti-IL-17 on CII stimulated $CD4^+$ T cell proliferation when dosed *in vivo* may be due to suppression of IL-1β and IL-6 production. The *in vitro* addition of anti-IL-17 had no effect on CII stimulated $CD4^+$ T cell proliferation suggesting, like IL-6, that IL-17 does not influence CII specific cells. However, the addition of anti-IL-1β *in vitro* was shown

to inhibit $CD4^+$ T cell proliferation. This indicates that the blockade of IL-17 in these conditions is not enough to inhibit IL-1β production, suggesting IL-1β can work independently of IL-17. Although the data reported here suggests anti-IL-17 cannot inhibit cell proliferation in an established immune response this cannot be completely ruled out *in vivo*.

Blocking IL-17 in CIA may also inhibit the destruction of cartilage and bone in the joint, which in turn may reduce the release of endogenous antigens such as CII that would normally drive the disease process. This may also apply to anti-TNFα and anti-IL-1β as their target cytokines have also been shown to play a role in bone erosion and cartilage degradation respectively (Bertolini *et al.*, 1986 and Shingu *et al.*, 1993).

In addition to all these biological actions, Ryu *et al.* (2006) showed that IL-17 was able to induce the expression of vascular endothelial growth factor (VEGF) from fibroblast-like synoviocytes, which is essential for angiogenesis in rheumatoid synovium and supports pannus formation. Another potential mode of action of anti-IL-17 may be the inhibition of angiogenesis, resulting in decreased pannus formation and indirectly reducing the number of cells being able to enter the joint. However, these potential late stage effects of anti-IL-17 would have no impact on CII immunity in the predictive models of CIA as joint damage and pannus formation has not occurred. The fact that disease is inhibited more profoundly by prophylactic anti-IL-17 treatment suggests that suppression of CII immunity and inflammation prevents joint destruction from ever occurring.

8.4.4 Value of the short-term models in identifying drug mechanisms of action

The use of these short-term readouts of CII immunity has identified the mechanisms of action of leflunomide and anti-CD40L and is consistent with data in the literature. These readouts also identified the mechanisms of action of anti-LFA-1 in CII immunity. However, it would appear that other factors can compensate for the blockade of LFA-1 in CIA. The assessment of the anti-cytokine drugs in these short-term models has identified both known and novel mechanisms of action. These data suggest that the short-term models and assays developed in this thesis have the potential to determine how a drug is exerting its anti-arthritic effect, something that cannot be achieved using CIA alone. Moreover, the differential effect of some of these drugs on CII induced hypersensitivity, anti-CII Ab production and CII stimulated $CD4^+$ T cell proliferation, suggests these readouts are capable of discriminating between drugs based on their mechanisms of action.

8.5 The role of Th1, Th2 and Th17 cells in CII immunity

To try to assess the role that IL-17 plays in CII immunity further, Chapter 7 looked at its relationship with other cytokines. This research arose because IL-17 was originally considered a Th1 cytokine detected in activated $CD4^+$ T cells (Yao *et al.*, 1995) but recent research has shown that IL-17 producing $CD4^+$ T cells represent a population of cells distinct from Th1 and Th2 subsets (Park *et al.*, 2005 and Harrington *et al.*, 2005). This has thrown into question the traditional Th1/Th2 paradigm that has been used to explain immune responses for a number of years. IL-17 does not just represent another novel cytokine but is also derived from a distinct new cell type, termed "Th17 cells", that may be pathogenic in a number of diseases. This is therefore an exciting time in immunology with the relationship between IL-17

secreting Th17 cells, IFNγ secreting Th1 and IL-4 secreting Th2 cells being unravelled. However, very little is known about IL-17 secreting Th17 cells in CIA and their relationship with Th1 and Th2 cells.

IL-17 is a key cytokine in CIA and in addition to the data in IL-17 deficient mice and blocking studies it was shown by Murphy *et al.* (2003) that mice deficient in IL-23, a cytokine involved in IL-17 production, are also protected against CIA. This was associated with an absence of IL-17 producing $CD4^+$ T cells. However, the role of IFNγ secreting Th1 cells in CIA is contradictory, with some researchers suggesting IFNγ exacerbates disease (Cooper *et al.*, 1988 and Boissier *et al.*, 1995) and others suggesting it protects against disease (Nakajima *et al.*, 1990 and Vermeire *et al.*, 1997).

In more recent years, a series of studies has put more weight on the fact that IFNγ is protective in CIA, as it has been shown that mice deficient in IFNγR develop a more severe form of disease than controls (Vermeire *et al.*, 1997, Manoury-Schwartz *et al.*, 1997 and Kelchtermans *et al.*, 2007). Furthermore, it has been demonstrated that mice, such as the C57B/6 strain, which are not normally susceptible to CIA, get disease when rendered deficient in IFNγ (Guedez *et al.*, 2001 and Chu *et al.*, 2007). In addition, mice deficient in IL-12 (IL-12p35 deficient mice), a macrophage derived cytokine responsible for inducing IFNγ production, develop severe CIA (Murphy *et al.*, 2003). This has lead to the general opinion that IFNγ is protective in CIA.

Studies conducted in IFNγ or IFNγR deficient mice have suggested that IFNγ may exert its protective effect by decreasing activated $CD4^+$ T cells (Chu *et al.*, 2000), IL-6 (Chu *et al.*, 2007), IL-1β (Guedez *et al.*, 2001) and IL-2 production (Vermeire *et al.*, 1997) as well as inhibiting the granulocyte chemotactic protein-2, a neutrophil attractant (Kelchtermans *et al.*, 2007). It has also been shown that IFNγ decreases IL-

23 and increases IL-12 production from infected DCs (Cruz *et al.*, 2006) therefore producing a cytokine milieu supportive of further IFNγ secretion. Furthermore, IFNγ has been shown to up-regulate IL-4 production (Vermeire *et al.*, 1997), IL-1Ra production (Tilg *et al.*, 1993) and support Treg cell activity (Kelchtermans *et al.*, 2005). Thus IFNγ has the potential to convey a strong inhibitory effect on immunity and inflammation.

Recently there have been a number of studies suggesting that the suppressive effect of IFNγ on the immune response is due to its influence on the production and development of IL-17 secreting Th17 cells. IFNγ has been shown to inhibit the differentiation of Th17 cells from naïve T cells and the production of IL-17 *in vitro* (Harrington *et al.*, 2005 and Park *et al.*, 2005) therefore suggesting IFNγ acts by reducing the activity of the pathogenic Th17 cell. In support of this, it has been shown that IFNγ and IL-12 deficient mice, which get severe arthritis, have an associated increase in IL-17 production (Chu *et al.*, 2007 and Murphy *et al.*, 2003). In addition, Nakae *et al.* (2007) has shown that IL-12 promotes Th1 cell differentiation but suppresses Th17 cell differentiation.

The role of IL-4 in CIA is more straightforward and has been shown to protect against CIA and joint damage when dosed systemically (Joosten *et al.*, 1999) with its blockade increasing disease activity (Yoshino. 1998). However, in the current work it would appear IL-4 secreting Th2 cells have little role to play in CII immunity as there was no detectable IL-4 produced in *ex vivo* cell cultures from CII sensitised mice in response to CII stimulation (Chapter 6). This difference may relate to the time of assessment. It may be that IL-4 is involved early on in the initial differentiation of Th subsets therefore blocking it could divert the immune response to a Th1/Th17

phenotype. This theory is supported by Yoshino (1998) who only administered anti-IL-4 for 10 days over the sensitisation period, suggesting an early action of this Ab. Further support of this theory comes from Harrington *et al.* (2005) who showed that IL-4 and therefore by inference Th2 cells are capable of inhibiting the formation of Th17 cells from naïve T cells in culture, which would have to occur early in the immune response. This suggests that if the suppressive effect of IL-4 were blocked an increase in IL-17 secreting Th17 cells may be expected. In addition, Park *et al.* (2005) has shown that anti-IL-4 increases the production of IL-17. The study by Yoshino (1998) also showed that the augmentation of CIA through IL-4 blockade was associated with an increase in IFNγ secretion and suggested that Th1 cells were responsible. However, the magnitude of the Th17 response was not assessed. Blocking IL-4 may therefore allow the development of both Th1 and Th17 cells with the latter being pathogenic and outnumbering the former, which is protective, resulting in arthritis. Without assessing IL-17 production it is easy to see why researchers conclude that IFNγ secreting Th1 cells are pathogenic. This is true for a number of studies that have identified Th1 cells as the dominant arthritogenic Th subset in CIA such as Mauri *et al.* (1996) and Doncarli *et al.* (1997). The blockade of both IFNγ and IL-4 *in vitro* has been shown to result in a synergistic increase in IL-17 production (Park *et al.*, 2005 and Chu *et al.*, 2007), again suggesting Th1 and Th2 cells can suppress Th17 cells.

In Chapter 7, it was shown that the addition of rmIL-4 *in vitro* caused an increase in the number of IFNγ secreting $CD4^+$ T cells in culture. As IL-4 appears to be protective in CIA this suggests that an increase in IFNγ may be a novel mechanism of action of IL-4 therapy. Although this goes against the general understanding that Th2

cells inhibit Th1 cell activity and vice versa (Fiorentino *et al.*, 1989 and Fitch *et al.*, 1993) there is evidence that in the right situation IFNγ can stimulate IL-4 production from Th2 cells (Bocek *et al.*, 2004). However, the current finding is in contrast to other CIA studies.

Kim *et al.* (2001) and Saidenberg-Kermanac'h *et al.* (2004) demonstrated that overexpression of IL-4 *in vivo* by transfected DCs or fibroblasts resulted in a decrease in IFNγ production and arthritis, and Yoshino (1998) showed that blockade of IL-4 enhanced IFNγ secretion. The discrepancy between the effects of IL-4 *in vitro*, as shown in this thesis, and data generated *in vivo* in the literature, suggests IL-4 may have additional effects *in vivo*. However, in all the *in vivo* studies, cells were taken for culture at a much later time point than Day 14 post-CII sensitisation and in the papers by Kim *et al.* (2001) and Saidenberg-Kermanac'h *et al.* (2004) spleen cell cultures were used. It may be that later on in the disease process IL-4 is capable of inhibiting the production of both IFNγ and IL-17 and is the key cytokine in the remission phase of disease. The difference between the actions of IL-4 *in vivo* as compared to *in vitro* could represent a difference in the time and site at which sampling was conducted.

IL-4 is a difficult cytokine to detect in *ex vivo* cell cultures from CIA mice but IL-10, another Th2 cytokine, appears to be increased late in disease, suggesting Th2 cells are up regulated and responsible for disease remission (Mauri *et al.*, 1996), which supports the theory that Th2 cells dominate at this stage. However, in these studies levels of IL-17 were not assessed and it would be interesting to see if IL-4 was able to suppress production of IL-17 in the later phase of disease.

It is possible that IL-4 has a biphasic effect in CIA, increasing IFNγ early in the disease process and then decreasing it in the later stages. The presence of both IFNγ and IL-4 may act synergistically to suppress IL-17 production as suggested by Park *et*

al. (2005), in the early stages of disease. This may be enough to inhibit development of CII immunity and hence CIA. Once the immune response is under control IL-4 may then suppress IFNγ and it is this reduction in IFNγ that is detected in the studies which overexpressed IL-4.

The data in this thesis has shown that CIA in DBA/1 mice is a Th17 driven response with IL-17 secreting $CD4^+$ T cells being detected prior to IFNγ secreting $CD4^+$ T cells. Although the relative amounts of cytokine cannot be used to determine their hierarchy, it is also interesting that higher levels of IL-17 were produced in response to CII than IFNγ. In Chapter 7, it was shown that these Th17 cells represent a very small population of $CD4^+$ T cells in CII immunity, which suggests they are potent pathogenic cells.

IFNγ secreting Th1 cells and IL-4 secreting Th2 cells can suppress the differentiation of naïve T cells into IL-17 producing Th17 cells. However, the work in Chapter 7 focused on the effect of these cytokines on committed CII specific $CD4^+$ T cells. Harrington *et al.*, (2005) had already reported that *in vitro* mature Th17 cells are resistant to Th1 and Th2 polarising conditions. The current work confirms and extends these findings, showing for the first time that *in vivo* derived CII specific IL-17 secreting $CD4^+$ T cells are not suppressed by IFNγ or IL-4. These findings are similar to those reported by Murphy *et al.*, (1996) for mature Th1 and Th2 cells, which are not reversed by the presence of IL-4 or IFNγ respectively.

In contrast to the current study and Harrington *et al.* (2005), Chu *et al.* (2007) demonstrated that IFNγ was capable of inhibiting IL-17 production in cultures of cells from CII sensitised mice, suggesting IFNγ can influence committed Th17 cells. The endpoints in these studies was however slightly different, with the current study

assessing the frequency of IL-17 secreting $CD4^+$ T cells by ELISpot. Harrington assessing the development of Th17 cells by FACS and Chu assessing the production of IL-17 in supernatants. The inhibitory effect of IFNγ on committed Th17 cells in terms of IL-17 production in the current study and in the Harrington paper can therefore not be ruled out.

The discrepancy that exists between the Chu study and this thesis may be because Chu used cells from C57B/6 mice deficient in IFNγ which have an altered immune system as compared to DBA/1 mice. It would appear that adding IFNγ to cell cultures where it is not naturally present is capable of inhibiting IL-17 production, as reported by Chu. Whereas, adding IFNγ to cell cultures where it is already present has no effect, as reported here. Chu also showed that blocking IFNγ in cell cultures from wild type C57B/6 mice, which were shown to have a dominant Th1 response, increased IL-17 production suggesting endogenous IFNγ is suppressing IL-17.

CII stimulated $CD4^+$ T cell cultures in this thesis had approximately 10 fold less IFNγ production as detected by ELISA than cell cultures from wild type mice in the Chu paper and appears not to be the dominant cytokine. In addition, blocking IFNγ in the ELISpot assay had no effect on the number of CII stimulated IL-17 secreting $CD4^+$ T cells in culture. There appears to be a fundamental difference between cells from the two mouse strains, where cells from C57B/6 mice genetically favour a Th1 response and those from DBA/1 mice favour a Th17 response. This may explain the differences between the studies.

Researchers have been focusing on the role that IFNγ secreting Th1 and IL-4 secreting Th2 cells have on the development and regulation of IL-17 producing Th17 cells, but have not addressed the effect IL-17 may have on Th1 or Th2 cells. In the

current study, results in Chapter 7 demonstrated that endogenous IL-17 in $CD4^+$ T cell cultures can suppress the formation of IFNγ secreting Th1 cells, but not IL-4 secreting Th2 cells. This is a novel finding and demonstrates that anti-IL-17 may exert part of its anti-arthritic effect by increasing the number of cells capable of secreting IFNγ. This infers that Th17 cells can suppress Th1 cells, so that in IL-17 dominant immune responses, such as CIA, Th1 cells and their associated protective actions are inhibited, giving rise to arthritis.

Nakae *et al.* (2007) showed in $CD4^+$ T cell cultures from ovalbumin (OVA) specific TCR transgenic mice deficient in IL-17 that the formation of Th1 cells was increased. However, in this study exogenous IL-12 was added to their cell cultures to force the differentiation of Th1 cells. In the absence of IL-12 it was shown in the same paper that cells from IL-17 deficient mice have a decreased number of Th1 cells as compared to wild type mice which suggests IL-17 does not suppress Th1 differentiation. In addition, these cultures used naïve T cells which have previously been shown to be susceptible to cytokine manipulation. The work carried out in this thesis has therefore shown for the first time in a disease model that endogenous IL-17 is capable of suppressing committed CII specific Th1 cells without any exogenous cytokine manipulation.

An increase in IFNγ secreting $CD4^+$ T cells appears to represent a novel mechanism of action for anti-IL-17. It is possible that this mechanism of action may also apply to anti-IL-6, anti-IL-1β and anti-TNFα as their target cytokines have been implicated in the development of Th17 cells. These drugs may therefore suppress Th17 cells and hence IL-17 production with an associated increase in IFNγ secreting Th1 cells. This may represent a common mechanism of action for these anti-cytokine therapies. To

test this theory the cytokine profiles from cell culture supernatants of *in vivo* drug treated mice could be assessed along with ELISpot assays. Data in this thesis has already shown that *in vivo* anti-IL-6 administration inhibits *ex vivo* CII stimulated IL-17 production, suggesting a role for IL-6 in the generation of Th17 cells. It would therefore be of interest to see if anti-IL-1β and anti-TNFα have a similar effect on IL-17 production and if these therapies increase the number of IFNγ secreting Th1 cells.

The *in vitro* addition of anti-IL-17 in the CII stimulated $CD4^+$ T cell proliferation assay had no effect. Although anti-IL-17 does not have an effect on CII specific $CD4^+$ T cell proliferation it may, in light of the current data, increase the number of IFNγ secreting Th1 cells. This may, in turn, alter the balance between Th1 and Th17 cells in culture, with no change in proliferation being detected. If this is the case it is unlikely that IFNγ is responsible for inhibiting the number of Th17 cells, as anti-IFNγ and rmIFNγ had no effect on the number of IL-17 secreting cells, although this cannot be completely ruled out as the cell concentrations varied between studies. Furthermore, it would be interesting to assess the cytokine profile in the supernatants of *in vitro* drug treated cultures to see if there is a change in the balance between IFNγ, IL-4 and IL-17.

The data presented in this thesis suggests that IL-17 secreting Th17 cells are the pathogenic subset of $CD4^+$ T cells in CIA. However, it still remains to be seen if IL-17 secreting Th17 cells have an important role to play in RA, but if they do, blocking IL-17 or depleting Th17 cells may represent an effective therapy.

8.6 Conclusion

The aim of this thesis, as outlined in the introduction, was to determine the anti-arthritic activity of a number of drugs in the CIA model and to development short-term models of CII immunity that can predict anti-arthritic drug activity. This has now been addressed.

It has been shown that short-term models of CII immunity are predictive of anti-arthritic drug effects in CIA. These short-term readouts may provide the basis of a novel screening cascade (Figure 8.1) for anti-arthritic drugs. Such a screening strategy has not been reported previously and has the potential to decrease the timeframe involved in the assessment of novel therapeutics. It may also lead to a decrease in the number of mice used as well as the number rendered arthritic, which has obvious welfare advantages.

In addition, these pre-arthritic readouts have been shown to be capable of discriminating between the mechanisms of action of anti-arthritic drugs, which may go some way to inform on where best to place novel therapies in the clinic. It has also been highlighted that the routine analysis of cytokine profiles in supernatants of CII stimulated $CD4^+$ T cell cultures may aid in this discrimination process.

The work has confirmed that CIA and CII immunity are $CD4^+$ driven responses. In addition, IL-17 has been shown to be a key cytokine in CII immunity and it appears that it is the Th17 subset of $CD4^+$ T cells that are the dominant and pathogenic cell type. Studies also demonstrated that IL-6 plays a pivotal role in IL-17 production in CII immunity, thus confirming its importance in Th17 cell development.

It has been shown for the first time that CII specific IL-17 secreting Th17 cells are capable of suppressing IFNγ secreting Th1 cells. In addition, it appears that these CII specific Th17 cells are resistant to IFNγ secreting Th1 and IL-4 secreting Th2 cells.

This supports the opinion that Th17 cells are disease promoting while Th1 cells are disease suppressing. However, the importance of IL-17 in the pathogenesis of human disease is as yet unknown, but with therapies fast approaching the clinic it is only a matter of time before this question is answered.

It will be of great interest to see if the novel therapies studied in this project have a beneficial impact in the clinical treatment of RA. The outcomes from the clinic will hopefully further support these short-term models as valuable tools in pre-clinical drug discovery.

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